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<b>(54) Title: KINASE RECEPTOR ACTIVATION ASSAY</b>			
<b>(57) Abstract</b>			
<p>An assay for measuring activation (i.e., autophosphorylation) of a tyrosine kinase receptor of interest is disclosed. a) A first solid phase is coated with a substantially homogeneous population of cells so that the cells adhere to the first solid phase. The cells have either an endogenous tyrosine kinase receptor or have been transformed with DNA encoding a receptor or "receptor construct" and the DNA has been expressed so that the receptor or receptor construct is presented in the cell membranes of the cells. b) A ligand is then added to the solid phase having the adhering cells, such that the tyrosine kinase receptor is exposed to the ligand. c) Following exposure to the ligand, the adherent cells are solubilized, thereby releasing cell lysate. d) A second solid phase is coated with a capture agent which binds specifically to the tyrosine kinase receptor, or, in the case of a receptor construct, to the flag polypeptide. e) The cell lysate obtained in step c) is added to the wells containing the adhering capture agent so as to capture the receptor or receptor construct to the wells. f) A washing step is then carried out, so as to remove unbound cell lysate, leaving the captured receptor or receptor construct. g) The captured receptor or receptor construct is exposed to a labelled anti-phosphotyrosine antibody which identifies phosphorylated residues in the tyrosine kinase receptor. h) Binding of the anti-phosphotyrosine antibody to the captured receptor or receptor construct is measured.</p>			

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KINASE RECEPTOR ACTIVATION ASSAYBACKGROUND OF THE INVENTIONField of the Invention

This invention relates to a kinase receptor activation (KIRA) assay. 5 In particular, the invention relates to an assay for measuring autophosphorylation of the kinase domain of a receptor protein tyrosine kinase (rPTK) using a kinase receptor activation, enzyme-linked immunosorbent assay (KIRA ELISA).

Description of Related Art

10 One mechanism for signal transduction in animals involves protein phosphorylation. Protein phosphorylation involves the action of protein kinase, an enzyme that transfers a phosphate group from a phosphate donor onto an acceptor amino acid in a substrate protein. Protein phosphatases provide a means for reversing the signal when the stimulus is removed.

15 Protein kinases have multiple substrates, and classification of the protein kinases is based on the acceptor amino acid specificity. The two most well characterized protein kinases are the protein kinases with a protein alcohol group as acceptor called protein serine/threonine kinases and the protein kinases with a protein phenolic group as acceptor called 20 protein tyrosine kinases (Hunter, Methods in Enzymology 200:3-9 [1991]).

The most well known type of signal-transducing protein kinases are growth factor receptor protein tyrosine kinases (rPTKs). rPTKs usually comprise a large, glycosylated, extracellular ligand binding domain (ECD) and an intracellular domain (ICD) which contains a tyrosine kinase catalytic domain. A single hydrophobic transmembrane (TM) domain connects the ECD and ICD. Examples of rPTKs include the insulin receptor, epidermal growth factor receptor (EGF-R), platelet-derived growth factor receptor (PDGF-R), insulin-like growth factor 1 receptor (IGF-1-R), and the HER2 receptor, to name a few. See, for example, Ullrich and Schlessinger Cell 25 61:203-212 (1990) and Fantl et al., Annu. Rev. Biochem. 62:453-481 (1993). rPTKs can phosphorylate exogenous protein substrates and intrinsic tyrosine residues via their catalytic tyrosine kinase domain. The intrinsic tyrosine residues normally reside in the ICD of the rPTK (see Figure 1 herein). Activation of the intracellular kinase domain of rPTKs appears 30 to be mediated by receptor oligomerization which results from the conformational alteration of the ECD upon ligand binding thereto. See 35

Ullrich and Schlessinger, *supra*.

Serine-threonine kinases have also been disclosed in the literature. While most of the known protein serine-threonine kinases are cytoplasmic proteins, a family of mammalian transmembrane receptors with serine-threonine kinase domains has recently been found. Members of this receptor family have been described as binding TGF- $\beta$ 's and activin. For reviews of serine-threonine kinases, see Sale, G., *Biochem. Soc. Transactions* 20: 664-670 (1992); ten Dijke et al., *Prog. in Growth Factor Res.* 5: 55-72 (1994); and Mathews, L., *Endoc. Rev.* 15(3): 310-325 (1994).

Various assays have been developed which measure tyrosine kinase activity. Some of these assays measure the ability of a tyrosine kinase enzyme to phosphorylate a synthetic substrate polypeptide. For example, an assay has been developed which measures growth factor-stimulated tyrosine kinase activity by measuring the ability of the kinase to catalyze the transfer of the  $\gamma$ -phosphate of ATP to a suitable acceptor substrate. See Pike, L., *Methods of Enzymology* 146:353-362 (1987) and Hunter, *Journal of Biological Chemistry* 257(9):4843-4848 (1982), for example. In this assay, the use of [ $\gamma$ - $^{32}$ P]ATP permits the radioactive labeling of the phosphorylated substrate, which is a synthetic tyrosine-containing peptide. Others have described protein kinase assays wherein incorporation of  $^{32}$ P into a tyrosine kinase receptor, such as the EGF receptor (see Donato et al., *Cell Growth Differ.* 3:259-268 [1992]), insulin receptor (see Kasuga et al., *Journal of Biological Chemistry* 257(17):9891-9884 [1982] and Kasuga et al., *Methods in Enzymology* 109:609-621 [1985]), and liver growth hormone receptor (see Wang et al., *Journal of Biological Chemistry* 267(24):17390-17396 [1992]), is measured.

The discovery of anti-phosphotyrosine antibodies has provided a non-radioactive, alternative means for measuring phosphorylation of tyrosine residues. For example, White and Backer (*Methods in Enzymology* 201:65-67 [1991]) mention polyclonal antibodies which selectively bind to phosphotyrosine and are considered to be useful for studying rPTKs. An anti-phosphotyrosine monoclonal antibody was used in one of the assays referred to in Madden et al. (*Anal. Biochem.* 199:210-215 [1991]), which measured phosphatase activity toward the insulin receptor. Anti-phosphotyrosine antibodies were also used by Cleaveland et al., in their protein tyrosine kinase ELISA assay. See Cleaveland et al., *Analytical Biochemistry* 190:249-253 (1990). The method of Cleaveland et al. utilizes purified high-activity oncogene tyrosine kinases, v-src and v-fps, and

measures the ability of these tyrosine kinases to phosphorylate synthetic polymeric substrates which are coated on an ELISA microtiter plate. The phosphotyrosine produced by src-induced phosphorylation of the polymeric substrate is then quantitated by addition of an anti-phosphotyrosine antibody, the presence of which is detected using a second rabbit anti-mouse antibody which is linked to a reporter enzyme, horseradish peroxidase (HRPO). A similar ELISA assay has been developed by Lazaro et al., which is used for detection of a protein tyrosine kinase. See Lazaro et al., Analytical Biochemistry 192:257-261 (1991). Like the assay of Cleaveland et al., this assay also measures the ability of a protein tyrosine kinase to phosphorylate a synthetic substrate which is bound to microELISA wells.

A direct way to assess specific activation of rPTKs is by analysis of receptor autophosphorylation. See, e.g., Hunter and Cooper Ann Rev Biochem 54:897-930 (1985) and Ullrich and Schlessinger, Cell 61:203-212 (1990). Using this direct approach, Knutson and Buck disclose assays for measuring autophosphorylation of the insulin receptor under *in situ* or *in vitro* conditions (Archives of Biochemistry and Biophysics 285(2):197-204 [1991]). In the *in situ* assay, monolayer cultures of embryonic mouse 3T3-C2 fibroblasts (having the endogenous insulin receptor) are incubated with insulin in large cell culture dishes. Following incubation, the insulin receptor is extracted from the membranes. To achieve extraction of the insulin receptor, the cell monolayers are scraped into a buffer containing protease inhibitors and the cells are then disrupted in a homogenizer. The cellular homogenate is subsequently subjected to centrifugation for 60 min., and the pellet which forms is extracted into buffer containing detergent. Following a further centrifugation step, the supernatant (containing the insulin receptor) is incubated with an anti-insulin receptor antibody. Then, the receptor-antibody complex is incubated with protein A-agarose and unoccupied protein A sites are blocked with normal rabbit IgG. The agarose beads are then centrifuged, the supernatants aspirated and the pellets are re-suspended in buffer containing the radiolabelled anti-phosphotyrosine antibody. The amount of bound iodinated anti-phosphotyrosine antibody is consequently measured.

Klein and his colleagues discuss an assay for measuring insulin activation of the insulin receptor (Klein et al., Diabetes 42:883-890 [1993]). In this assay, aliquots of a heterogeneous population of mononuclear blood cells (including T cells, B cells, macrophages etc) having the insulin receptor are exposed to insulin in centrifuge tubes.

The cells are then lysed in detergent using a motordriven homogenizer and the lysates are concentrated two- to fourfold using vacuum centrifugation. Sometimes, the insulin receptor is also partially purified using wheat germ agglutin agarose. The supernatants which form following centrifugation, 5 are then transferred to anti-insulin receptor-coated microtiter plates. Insulin (8.7nM) as well as kinase and phosphatase inhibitors are present during receptor immobilization in order to optimize the percentage of receptors captured to the microtiter plates. Activation of the insulin receptor is then measured by transphosphorylation of the substrate Poly- 10 Glu,Tyr with  $^{32}$ P labeled ATP. The supernatants are then spotted onto absorbent paper and the paper is washed with cold TCA to remove unbound  $^{32}$ P-ATP. Remaining  $^{32}$ P-labeled Poly-Glu,Tyr on the washed absorbent paper is subsequently counted by scintillation counting.

Hagino et al. were also interested in studying the insulin receptor 15 in patients (Hagino et al., Diabetes 43:274-280 [1994]). As a first step in the assay, Hagino et al. stimulate a primary cell suspension, which is not particularly homogeneous with respect to cell type. In particular, heparinized blood (1ml washed twice with medium and resuspended in 1 ml of medium containing bovine serum albumin, BSA) is exposed to varying 20 concentrations of insulin. The autophosphorylation reaction is stopped, the cells centrifuged for 30 min, the supernatant is discarded and the erythrocyte ghosts thus obtained are resuspended in buffer and centrifuged again. The pellet thereby obtained is adjusted to 500 $\mu$ l and solubilized 25 in detergent. The solubilized materials are then centrifuged and the resulting supernatant is subjected to sandwich ELISA (using anti-insulin receptor antibodies to capture the insulin receptor) to determine the extent of insulin receptor autophosphorylation.

King et al. in Life Sciences 53: 1465-1472 (1993) describe a colorimetric assay for examining inhibitors of the epidermal growth factor 30 (EGF) receptor-associated tyrosine kinase in human intact epidermal A431 cells.

Several others have used an enzyme-conjugated form of the anti-phosphotyrosine antibody in Western blot analyses which measure receptor autophosphorylation. Briefly, Western blotting generally involves 35 electrophoresing activated rPTK on polyacrylamide gel. The rPTK is then transferred to nitrocellulose and immunoblotted with the anti-phosphotyrosine antibody which is labelled to enable detection. See, for example, Wang, Molecular and Cellular Biology 5(12):3640-3643 (1985);

Glenney et al., Journal of Immunological Methods 109:277-285 (1988); Kamps, Methods in Enzymology 201:101-110 (1991); Kozma et al., Methods in Enzymology 201:28-43 (1991); Holmes et al., Science 256:1205-10 (1992); and Corfas et al., PNAS, USA 90:1624-1628 (1993). However, with Western blot analysis, accurate quantitation can be very tedious. Furthermore, this technique tends to be time-consuming and generally does not allow high sample throughput.

It is an object of the instant invention to provide a sensitive, reliable assay that measures receptor protein tyrosine kinase (rPTK) 10 autophosphorylation. The assay is desirably useful for qualitatively and quantitatively measuring kinase activation as well as facilitating identification and characterization of potential agonists and antagonists for a selected rPTK. It is a further object of the invention to provide an assay which enables ligand-receptor interactions to be studied for any 15 selected rPTK.

This assay must have a capacity for high throughput, that is, the ability to reliably evaluate large numbers of samples in a relatively short period of time (e.g., in one day). The assay ideally does not use radioactive materials and is also amenable to automation.

It is a further object, in at least one embodiment of the invention, 20 to provide a generic assay which enables a rPTK of interest to be studied, regardless of whether or not a receptor-specific capture agent having the desired characteristics is available. Furthermore, it is an object of the invention to provide an assay which substantially represents the activity 25 of the tyrosine kinase receptor *in situ*. This is desirable insofar as it reduces the possibility that altered interactions between the receptor and the ligand may occur as a consequence of the receptor not being membrane-bound. Furthermore, if the receptor is a multimeric complex, this assay enables the correctly assembled receptor to be studied. It is an 30 additional object to provide a method for measuring serine-threonine kinase phosphorylation, phosphorylation of intracellular kinases and phosphatase activity.

These and other objects will be apparent to the ordinary artisan upon consideration of the specification as a whole.

Accordingly, the invention provides an assay for measuring activation (i.e., autophosphorylation) of a tyrosine kinase receptor of interest.

The assay can be divided into two major stages, each of which is generally performed in separate assay plates. The first stage of the assay involves activating the receptor and is termed the kinase receptor activation (KIRA) stage of the assay. The second stage of the assay 5 involves measuring receptor activation. Conveniently, this is achieved using an enzyme-linked immunosorbent assay (ELISA) to measure receptor activation.

The KIRA stage of the assay involves activating a tyrosine kinase receptor which is located in the cell membrane of an eukaryotic cell such 10 that the extracellular domain of the receptor faces the external milieu of the cell, the transmembrane domain is located in the cell membrane and the kinase domain is located intracellularly. This stage of the overall assay involves steps (a) to (c) below:

(a) The first solid phase (e.g., a well of a first assay plate) is 15 coated with a substantially homogeneous population of cells (usually a mammalian cell line) so that the cells adhere to the solid phase. Often, the cells are adherent and thereby adhere naturally to the first solid phase. In one embodiment of the invention, the cells have an endogenous tyrosine kinase receptor presented in the cell membrane as discussed above. 20 In an alternative embodiment, the cells have been transformed with DNA encoding a tyrosine kinase receptor or a "receptor construct" defined further below, which DNA is expressed by the cells such that the receptor or receptor construct is suitably positioned in the cell membranes thereof.

The receptor construct comprises a fusion of a kinase receptor and 25 a flag polypeptide. The flag polypeptide is recognized by the capture agent, often a capture antibody, in the ELISA part of the assay. Use of a receptor construct as disclosed herein is particularly advantageous since it provides a "generic" assay wherein autophosphorylation of any tyrosine kinase receptor can be measured, regardless of whether or not a receptor- 30 specific capture agent having the required characteristics is available. Often, the tyrosine kinase receptor is a fusion protein comprising the ECD of a selected tyrosine kinase and the catalytic ICD (and possibly the transmembrane domain) of another well characterized tyrosine kinase (e.g., the Rse receptor).

35 (b) An analyte is then added to the wells having the adhering cells, such that the tyrosine kinase receptor is exposed to (or contacted with) the analyte. This assay enables identification of agonist and antagonist ligands for the tyrosine kinase receptor of interest. In order to detect

the presence of an antagonist ligand which blocks binding and/or activation of the receptor by an agonist ligand, the adhering cells are exposed to the suspected antagonist ligand first and then to the agonist ligand (or to a mixture of the agonist and antagonist) so that competitive inhibition of 5 receptor binding and activation can be measured. Also, the assay can identify an antagonist which binds to the agonist ligand and thereby reduces or eliminates its ability to bind to, and activate, the rPTK. To detect such an antagonist, the suspected antagonist and the agonist for the rPTK are incubated together and the adhering cells are then exposed to this 10 mixture of ligands.

(c) Following exposure to the analyte, the adhering cells are solubilized using a lysis buffer (which has a solubilizing detergent therein) and gentle agitation, thereby releasing cell lysate which can be subjected to the ELISA part of the assay directly, without the need for 15 concentration or clarification of the cell lysate. Thus, this assay provides a significant improvement over assays described by Knutson and Buck, *supra*, Klein et al., *supra*, and Hagino et al., *supra*, insofar as it is surprisingly unnecessary to concentrate the cell lysate prior to the ELISA. Furthermore, unlike the other assays, in the instant assay the cells 20 can be lysed in lysis buffer using gentle agitation without the need for homogenizing, centrifuging or clarifying the cells. The cell lysate thus prepared is then ready to be subjected to the ELISA stage of the assay. It has been discovered that, surprisingly, the first assay plate can be stored at freezing temperatures (i.e., at about -20° to -70°C) for significant 25 periods of time (at least 6 months) before the ELISA stage of the assay. This is a significant finding insofar as the KIRA and ELISA stages of the assay can be performed on separate days.

The ELISA component of the assay comprises steps (d) to (h), described below.

30 (d) As a first step, the second solid phase (usually a well of an ELISA microtiter plate) is coated with a capture agent (often a capture antibody) which binds specifically to the tyrosine kinase receptor, or, in the case of a receptor construct, to the flag polypeptide. Coating of the second solid phase is carried out so that the capture agent adheres to the 35 second solid phase. The capture agent is generally a monoclonal antibody, but, as is described in the examples herein, polyclonal antibodies may also be used.

(e) The cell lysate obtained in step (c) of the above-mentioned KIRA stage of the assay is exposed to, or contacted with, the adhering capture agent so that the receptor or receptor construct adheres to (or is captured in) the second solid phase. Unlike the assay of Klein et al., the instant 5 assay does not require the ligand for the receptor as well as kinase inhibitors to be present to achieve suitable immobilization of the receptor or receptor construct to the second solid phase.

(f) A washing step is then carried out, so as to remove unbound cell lysate, leaving the captured receptor or receptor construct.

10 (g) The adhering or captured receptor or receptor construct is then exposed to, or contacted with, an anti-phosphotyrosine antibody which identifies phosphorylated tyrosine residues in the tyrosine kinase receptor. In the preferred embodiment, the anti-phosphotyrosine antibody is conjugated (directly or indirectly) to an enzyme which catalyses a color 15 change of a non-radioactive color reagent. Accordingly, phosphorylation of the receptor can be measured by a subsequent color change of the reagent. The enzyme can be bound to the anti-phosphotyrosine antibody directly, or a conjugating molecule (e.g., biotin) can be conjugated to the anti-phosphotyrosine antibody and the enzyme can be subsequently bound to the 20 anti-phosphotyrosine antibody via the conjugating molecule.

(h) Finally, binding of the anti-phosphotyrosine antibody to the captured receptor or receptor construct is measured, e.g., by a color change in the color reagent.

The invention also pertains to a Rse.flag reagent which is 25 particularly useful for use in the KIRA ELISA assay. The Rse.flag reagent is a polypeptide comprising a fusion of a flag polypeptide (usually the gD flag described herein) to the carboxyl terminus of the intracellular domain of the Rse rPTK. Generally, the transmembrane domain of Rse and the extracellular domain of another rPTK of interest are also present in the 30 fusion polypeptide reagent. The nucleic acid encoding this reagent and a cell transformed therewith are also claimed.

In yet a further aspect, the invention relates to a kit which can be used in the KIRA ELISA disclosed above which comprises an anti-flag polypeptide capture agent (e.g. a capture antibody) which is usually bound 35 to the second solid phase as described herein. Thus, the kit generally provides an ELISA microtiter plate having an anti-flag polypeptide capture antibody adhering to a well thereof. Optionally, the kit also provides an anti-phosphotyrosine antibody which is often labelled, or reagents for

labelling the anti-phosphotyrosine antibody are supplied with the kit. Sometimes, a homogeneous population of cells which have been transformed with a receptor construct as described herein are also provided with the kit. The kit also suitably includes instructions for carrying out the KIRA  
5 ELISA.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1C are diagrammatic representations of Rse.gD (Figure 1A), Receptor ECD/Rse.gD chimera (Figure 1B) and a CHO cell transformed with the  
10 Receptor ECD/Rse.gD chimera (Figure 1C).

Figures 2A and 2B depict an alignment of the amino acid sequence (SEQ ID NO: 1) and nucleotide sequence (SEQ ID NO: 2) of Rse.gD. The residues of the signal sequence are indicated with an (\*), the transmembrane domain of Rse is boxed and the ECD and ICD of Rse are also delineated. The  
15 residues of the gD flag sequence are underlined.

Figure 3 is a flow diagram of an exemplary strategy for selecting a suitable capture agent for use in the assay.

Figure 4 is a flow diagram of an exemplary strategy for selecting a transformed cell suitable for use in the assay, where the cell has a receptor construct with an amino-terminal flag polypeptide located in the cell membrane thereof.  
20

Figure 5 is a flow diagram of an exemplary strategy for selecting a transformed cell suitable for use in the assay, where the cell has a receptor construct with a carboxyl-terminal flag polypeptide located in the cell membrane thereof.  
25

Figure 6 is a flow chart and cartoon illustrating the KIRA ELISA assay for the HER2 receptor described in Example 1.

Figure 7 depicts a p185<sup>HER2</sup>/HRG $\beta$ 1<sub>177-244</sub> KIRA ELISA standard curve obtained using the assay described in Example 1. To obtain the standard  
30 curve, MCF-7 cells ( $2 \times 10^5$ ) were stimulated with 3000, 1000, 333, 111, 37, 12, 4, or 0 pM HRG $\beta$ 1<sub>177-244</sub>, as determined by quantitative amino acid analysis (q.a.a.a.). Each calibrator concentration was run in triplicate. The values derived from 10 such standard curves were averaged (total n = 30) and are presented as mean ABS<sub>450/650</sub> ± sd vs. HRG $\beta$ 1<sub>177-244</sub> concentration.

Figure 8 depicts heregulin specificity of p185<sup>HER2</sup>/HRG KIRA ELISA of Example 1. In the assay, MCF-7 cells ( $2 \times 10^5$ ) were stimulated with either HRG $\beta$ 1<sub>177-244</sub> (■) at 3000, 1000, 333, 111, 37, 12, 4 or 0 pM or IGF-1(▲), EGF (□), VEGF (●) or insulin (♦) at 30000, 10000, 3333, 1111, 370, 120, 40 or  
35

0 pM. For all concentrations of ligands, n = 3 and data are presented as average ABS<sub>450/650</sub> ± sd vs. ligand concentration.

Figure 9 is a flow chart and cartoon illustrating the KIRA ELISA assay for the Rse receptor described in Example 2.

5 Figure 10 depicts a Rse KIRA ELISA standard curve obtained using the assay described in Example 2. To obtain the standard curve, CHO cells transformed with the Rse.gD construct were stimulated with 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200 or 0 diluted, anti-Rse agonist antibody. Each calibrator concentration was run in triplicate. The values are presented  
10 as mean ABS<sub>450/650</sub> ± sd vs. 1/dilution agonist antibody.

Figure 11 is a flow chart and cartoon illustrating the KIRA ELISA assay for the trk receptors (i.e., trk A, trk B, and trk C) described in Example 3.

Figures 12A-12D depict an alignment of the amino acid sequence  
15 (SEQ ID NO: 3) and nucleotide sequence (SEQ ID NO: 4) of gD.trk A used in the assay described in Example 3. The residues of the signal sequence are indicated with an (\*), the residues of the gD flag sequence are underlined, the residues of the transmembrane domain of trk A are in bold and the ECD and ICD thereof are also delineated.

20 Figures 13A-13D depict an alignment of the amino acid sequence (SEQ ID NO: 5) and nucleotide sequence (SEQ ID NO: 6) of gD.trk B used in the assay described in Example 3. The residues of the signal sequence are indicated with an (\*), the residues of the gD flag sequence are underlined, the residues of the transmembrane domain of trk B are in bold and the ECD  
25 and ICD thereof are also delineated.

Figures 14A-14D depict an alignment of the amino acid sequence (SEQ ID NO: 7) and nucleotide sequence (SEQ ID NO: 8) of gD.trk C used in the assay described in Example 3. The residues of the signal sequence are indicated with an (\*), the residues of the gD flag sequence are underlined,  
30 the residues of the transmembrane domain of trk C are in bold and the ECD and ICD thereof are also delineated.

Figures 15A-15C depict standard curves for trk A, B and C, respectively, which were obtained using the assay described in Example 3. To obtain the standard curves, CHO cells transformed with the gD.trk constructs were stimulated with 3000, 1000, 333, 111, 37, 12, 4 or 0 pM of ligand, i.e. nerve growth factor (NGF, ■), neurotrophin 3 (NT3, ●) or neurotrophin 5 (NT5, ▲). The values are presented as mean ABS<sub>450/650</sub> ± sd vs. ligand concentration.

Figures 16A-16L depict the nucleotide sequence (SEQ ID NO: 9) of the pSVI17.ID.LL expression vector used for expression of Rse.gD in Example 2.

Figure 17 is a diagrammatic representation of the MPL/Rse.gD chimeric receptor described in Example 4.

5 Figure 18 is a flow chart and cartoon illustrating the KIRA ELISA for the MPL/Rse.gD chimeric receptor described in Example 4.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

##### I. Abbreviations and Definitions

"rPTK" means a receptor protein tyrosine kinase.

10 "ECD", "TM domain" and "ICD" refer to the extracellular domain, transmembrane domain and intracellular domain of a rPTK, respectively.

"Kinase Receptor Activation" or "KIRA" when used throughout this application refers to the first stage of the instantly claimed assay wherein a cell-bound rPTK is exposed to a potential agonist/antagonist 15 ligand which may (or may not) induce phosphorylation of tyrosine residues in the intracellular domain of the rPTK. The KIRA is generally carried out in the "first assay plate" as defined herein.

"Enzyme-Linked Immunosorbent Assay" or "ELISA" refers to the second stage of the instantly claimed assay and involves measuring tyrosine 20 phosphorylation of the rPTK. The ELISA is normally carried out in the "second assay plate" as disclosed in this application. The ELISA is a "sandwich ELISA" insofar as it involves capturing the rPTK or receptor construct to the second solid phase (usually the well of an ELISA microtiter plate). ELISA assays generally involve the preparation of 25 enzyme-antibody conjugates. The conjugated enzyme cleaves a substrate to generate a colored reaction product that can be detected spectrophotometrically. In this assay, the absorbance of the colored solution in individual microtiter wells is proportional to the amount of phosphotyrosines. A review of ELISA is found in Current Protocols in 30 Molecular Biology, Vol. 2, chapter 11 (1991). While the term "ELISA" is used to describe the second stage of the instant assay, it is only a preferred embodiment of the invention, since, as disclosed herein, techniques other than enzymatic detection are available for measuring binding of the anti-phosphotyrosine antibody to the activated receptor.

35 The terms "receptor", "kinase receptor", "tyrosine kinase", "tyrosine kinase receptor", "receptor protein tyrosine kinase" and "rPTK" are used

interchangeably herein and refer to a protein having at least one phosphate accepting phenolic group. The protein is usually a receptor insofar as it has a ligand-binding ECD, TM domain and ICD. The ICD usually comprises a catalytic kinase domain and has one or more phosphate accepting tyrosine residues. See Figures 1A and 1B, for example. Examples of tyrosine kinase receptors include the insulin receptor, insulin related receptor, epidermal growth factor receptor (EGF-R), platelet-derived growth factor receptors A and B (PDGF-R-A and PDGF-R-B), insulin-like growth factor 1 receptor (IGF-1-R), macrophage colony-stimulating factor receptor (M-CSF-R), HER2/neu/c-erbB-2 receptor, HER3/c-erbB-3 receptor, Xmrk receptor, IRR receptor, fibroblast growth factor (FGF) receptors bek and flg, c-kit receptor, Flk/kDR receptor, Rse receptor, the Eph, Elk, Eck, Eek, Erk, Cek4/Mek4/HEK and Cek5 receptors, Axl receptor, hepatocyte growth factor receptor (HGF-R), Flt1 VEGF receptor, SAL-S1 receptor, HpTK 5 receptor, trkA receptor, trkB receptor, and trkC receptor. See, for example, Ullrich and Schlessinger Cell 81:203-212 (1990); Fantl et al., Annu. Rev. Biochem. 62:453-481 (1993); Mark et al., Journal of Biological Chemistry 269(14):10720-10728 (1994); and WO 93/15201.

The terms mentioned above encompass chimeric "receptor" molecules which comprise at least the extracellular domain of a selected tyrosine kinase and the intracellular domain, and optionally, the transmembrane domain of another tyrosine kinase. Of course, the tyrosine kinase of interest can provide the transmembrane domain and/or intracellular domain. The terms also encompass amino acid sequence variants and covalent derivatives of the various rPTKs provided they still display tyrosine kinase phosphorylation activity in the KIRA ELISA. Therefore, the variants will generally have conservative amino acid alterations. The individual domains of the tyrosine kinase can be delineated based on sequence homology to known tyrosine kinases and hydrophobicity plots. For example, the hydrophobic transmembrane domain can be readily determined and the ECD and ICD are usually amino-terminal and carboxyl terminal to the transmembrane domain, respectively. Conveniently, the transmembrane domain and ICD of the Rse receptor can be fused to the ECD of a tyrosine kinase of interest, thereby forming a chimeric receptor which is encompassed by the terms denoting a receptor as mentioned above.

In the preferred embodiment, the rPTK is selected from the group consisting of HER2 receptor (Ullrich and Schlessinger, *supra*), Rse receptor

(Mark et al., *supra* and SEQ ID NO: 1), trk A receptor (SEQ ID NO: 3), trk B receptor (SEQ ID NO: 5) and trk C receptor (SEQ ID NO: 7).

By "autophosphorylation" is meant activation of the catalytic kinase domain of the rPTK, whereby at least one intrinsic tyrosine residue is 5 phosphorylated. Generally, autophosphorylation will result when an agonist molecule binds to the extracellular domain of the kinase receptor. Without being limited to any particular mechanism of action, it is thought that binding of the agonist molecule may result in oligomerization of the kinase receptor which causes activation of the catalytic kinase domain.

10 By "solid phase" is meant a non-aqueous matrix to which the cells (in the KIRA stage of the assay) or the capture agent (in the ELISA stage of the assay) can adhere. Usually, the solid phase comprises the well of an assay plate but the invention is by no means limited to this embodiment. For example, the solid phase can comprise a discontinuous solid phase of 15 discrete particles. The particles can be porous and formed from a number of different materials, e.g., polysaccharides (e.g. agarose), polyacrylamides, polystyrene, polyvinyl alcohol, silicones and glasses. For examples of suitable particulate solid phases, see U.S. Patent No. 4,275,149.

20 By "well" is meant a recess or holding space in which an aqueous sample can be placed. The well is provided in an "assay plate". The invention usually employs a "first assay plate" which is formed from a material (e.g. polystyrene) which optimizes adherence of cells (having the receptor or receptor construct) thereto. Generally, the individual wells 25 of the first assay plate will have a high surface area to volume ratio and therefore a suitable shape is a flat bottom well (where the cells are adherent). The "second assay plate" is generally formed from a material (e.g. polystyrene) which optimizes adherence of the capture agent thereto. The second assay plate may have the same general construction and/or 30 characteristics as the first assay plate. However, separate plates are used for the KIRA stage of the assay and the ELISA stage of the assay.

In the preferred embodiment of the invention, both the first assay plate and the second assay plate are "microtiter" plates. The term "microtiter" plate when used herein refers to an 35 assay plate having between about 30 to 200 individual wells, usually 96 wells. Often, the individual wells of the microtiter plate will hold a maximum volume of about 250 $\mu$ l. Conveniently, the first assay plate is a 96 well polystyrene or plastic, cell culture microtiter plate (such as that

sold by Becton Dickinson Labware, Lincoln Park, NJ), which allows for automation. Often, about  $50\mu\text{l}$  to  $300\mu\text{l}$ , more preferably  $100\mu\text{l}$  to  $200\mu\text{l}$ , of an aqueous sample comprising cell culture media with the cells suspended therein will be added to each well of the first assay plate in the KIRA 5 stage of the assay. It is desirable to seed between about  $1 \times 10^4$  to  $3 \times 10^5$  cells per well. More preferably,  $5 \times 10^4$  to  $1 \times 10^5$  cells per well are seeded. Usually, the second assay plate will comprise a polystyrene microtiter ELISA plate such as that sold by Nunc Maxisorp, Inter Med, Denmark.

10 The term "homogeneous population of cells" refers to a substantially homogeneous population of cells wherein at least about 80%, and preferably about 90%, of the cells in the population are of the same cell type. Therefore, it is convenient to use a cell line. The cell line is a eukaryotic cell line, normally an animal cell line and desirably a 15 mammalian cell line.

The cells have, or are transformed to produce, the selected receptor or a receptor construct. For example, where the kinase receptor is known to be present in a certain cell line (e.g., the HER2 receptor in the MCF-7 cell line) no transformation step is required. Conversely, it may be 20 necessary to transform a cell with a nucleic acid encoding the receptor, where the cell does not make the receptor, or does not have suitable numbers of the receptor in the cell membrane thereof. Accordingly, the cell is transformed with a nucleic acid encoding the receptor (or receptor construct) and the nucleic acid is expressed so that the ECD of the 25 receptor faces the external milieu of the cell, the transmembrane domain is located in the cell membrane and the kinase domain is located intracellularly.

Where the assay relies on activating the endogenous rPTK, a cell line is selected which is known to produce the rPTK of interest, provided 30 sufficient levels of the rPTK are present in the cell membrane thereof to enable detection. As a general proposition, a minimum number of about  $1 \times 10^4$  receptors/cell is required. For example, the MCF-7 cell line (ATCC-HTB 22) which produces the HER2 receptor was shown to be useful in the assay. There are  $5 \times 10^4$  HER2 receptors/MCF-7 cell. Examples of other cell lines 35 and their respective rPTKs include, embryonic mouse 3T3-C2 fibroblast cell line and the insulin receptor, and Hep 3B (ATCC # HB 8064) cell line and the Rse receptor. However, the degree of expression of the rPTK nucleic acid in the cell line is not so high that it results in constitutive

phosphorylation of the rPTK. For example, the SK-BR-3 cell line (ATCC HTB30), which has  $3 \times 10^6$  HER2 receptors/cell, was found to be unsuitable for use in the assay disclosed herein. Therefore, it may be useful to use a cell line which has less than about  $3 \times 10^6$  receptors/cell, depending on 5 the type of receptor. The number of receptors/cell can be measured using Scatchard analysis, for example (Scatchard, Ann. NY Acad. Sci. 51:660-672 [1949]; and Goodwin et al., Cell 73:447-456 [1993]). However, selection of a cell line having a suitable number of receptors/cell is possible using the techniques described herein.

10 The term "adherent" when used herein to describe the cell, refers to a cell which naturally adheres to the first solid phase (often the well of the first assay plate), thereby forming a fairly uniform coating of the cells on the inside surface of the well. The uniform coating of cells generally forms following incubation of the cells in the wells of the first 15 assay plate for about 8-16 hours. After incubation, non-adhering cells and cell culture medium are decanted off the first assay plate. Incubation is usually carried out at a temperature which is optimal for cell growth, i.e., about 37°C. Examples of adherent cell lines include CHO cells (Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216 [1980]), MCF-7 cells (ATCC HB 20 22), 293 cells (Graham et al., J. Gen Virol. 36:59 [1977]), Swiss albino 3T3 fibroblast cell line (ATCC No. CCL 92) and U937 macrophage cell line (ATCC No. CRL 1593).

A "flag polypeptide" comprises a short polypeptide which has enough residues to provide an epitope (preferably a linear epitope) against which 25 a "capture agent" thereagainst can be made, yet is short enough such that it does not interfere with activity of the rPTK. The flag polypeptide is also sufficiently unique so that the capture agent thereagainst does not bind to other reagents in the assay. Selection of a "unique" flag polypeptide sequence can be accomplished by comparing the sequence of a 30 proposed flag polypeptide against other known sequences in Genbank or EMBL, for example. Suitable flag polypeptides generally have at least 6 amino acid residues and usually between about 8-80 amino acid residues (preferably between about 9-30 amino acid residues).

By "receptor construct" is meant a polypeptide which comprises a 35 fusion of a kinase receptor and a flag polypeptide as defined above. The flag polypeptide is provided at a location in the receptor construct such that: a) the flag polypeptide does not interfere with ligand binding to the receptor; b) the flag polypeptide does not interfere with

autophosphorylation of the receptor and c) the flag polypeptide is presented in a suitable configuration so that it can bind to the capture agent in the ELISA stage of the assay. Often, the polypeptide flag will be present at the N-terminus of the receptor construct. See, for example, 5 Example 3 which refers to the gD.trk constructs. Alternatively, the flag polypeptide may be present at the C-terminus of the receptor construct. See, for example, Example 2 which refers to the Rse.gD construct. See also Figures 1A-1C. The Rse construct disclosed herein is particularly useful, since the ICD (and optionally the transmembrane domain) thereof can be 10 fused to the ECD of a kinase receptor of interest, thereby obviating the need to establish where the flag polypeptide should be located with respect to the kinase receptor of interest.

"Rse.gD" refers to a receptor construct which is the Rse receptor protein tyrosine kinase with the Herpes Simplex virus glycoprotein D (gD) 15 flag polypeptide fused to the COOH-terminus thereof.

"Rse.flag reagent" refers to a polypeptide which comprises the ICD of the Rse receptor fused at its COOH-terminus to a flag polypeptide (normally the gD flag polypeptide). Sometimes, the TM domain of Rse and the ECD of a rPTK of interest will also be present in the Rse.gD. reagent. 20 "Receptor ECD/Rse.gD Chimera" refers to a fusion of the ECD of a rPTK of interest to the TM and ICD domains of Rse which are fused COOH-terminally to the gD flag polypeptide.

"gD.trkA", "gD.trkB" and "gD.trkC" refer to each of the trk receptors (A-C) having the gD flag polypeptide fused to the amino-termini thereof. 25

By "capture agent" is meant a compound or agent which is able to adhere to the second solid phase, as herein defined, and which is selective for a rPTK or receptor construct. Thus, the capture agent captures the receptor or receptor construct to the wells of the second assay plate. Usually, the capture agent binds selectively to the flag polypeptide which 30 has been fused to the receptor of interest. Binding of the capture agent is not affected by the presence or absence of ligand bound to the receptor and does not induce receptor activation upon capture. Furthermore, the capture agent does not sterically block access to the phosphorylated tyrosine(s) by the anti-phosphotyrosine antibody. Means for selecting 35 suitable capture agents are described herein. Generally, the capture agent will comprise an antibody (e.g., an affinity purified polyclonal antibody or a monoclonal antibody), but other selective agents, such as streptavidin which binds selectively to the "strep-tag" polypeptide can also be used

(see Schmidt et al., Protein Engineering 6(1):109-122 [1993]). Streptavidin can be purchased commercially from Zymed Laboratories, S. San Francisco, CA, for example. Alternatively, the capture agent can comprise protein A (which binds specifically to immunoglobulins). In this 5 embodiment of the invention, the activated receptor or receptor-construct present in the cell lysate is incubated with an antibody which binds specifically thereto, thereby forming a receptor-antibody complex. This complex can be captured by protein A by virtue of its specific binding to the antibody present in the complex. Protein A can be purchased 10 commercially from Pharmacia Biotech, Inc., Piscataway, New Jersey, for example. A strategy for selecting a suitable capture agent is depicted in Figure 3 and will be described in more detail later herein.

In the most preferred embodiment, the capture agent is a monoclonal antibody which binds specifically to a flag polypeptide (which is present 15 in the receptor construct). Examples of suitable flag polypeptides and their respective capture antibodies include the flu HA flag and its antibody 12CA5, (Field et al., Mol. Cell. Biol. 8:2159-2165 [1988]); the c-myc flag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan et al., Molecular and Cellular Biology 5(12):3610-3616 [1985]); as well as 20 the Herpes Simplex virus glycoprotein D (gD) flag and the 5B6 antibody thereto (Paborsky et al., Protein Engineering 3(6):547-553 [1990] and Mark et al., Journal of Biological Chemistry 269(14):10720-10728 [1994]). Other flag polypeptides have been disclosed. Examples include the Flag-peptide 25 (Hopp et al., BioTechnology 6:1204-1210 [1988]); the KT3 epitope peptide (Martin et al., Science 255:192-194 [1992]); an  $\alpha$ -tubulin epitope peptide (Skinner et al., J. Biol. Chem. 266:15163-15166 [1991]); and the T7 gene 10 protein peptide tag (Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA 87:6393-6397 [1990]). Once the flag polypeptide has been selected as discussed above, a capture antibody thereto can be generated using the 30 techniques disclosed herein.

The term "analyte" refers to a compound or composition to be studied, usually to investigate its ability to activate (or prevent activation of) the tyrosine kinase receptor of interest. The analyte can comprise a bodily fluid (such as plasma or amniotic fluid) or a composition known to 35 contain, or suspected of containing, a ligand for the tyrosine kinase receptor. The analyte can also comprise a cell which has a ligand to the rPTK of interest.

"Ligand" when used herein refers to a molecule which is able to bind to the ECD of the tyrosine kinase of interest or to a known agonist for the tyrosine kinase of interest. The ligand will usually be an agonist or antagonist for the tyrosine kinase.

5 By "agonist" is meant a molecule which is able activate the intracellular kinase domain of the tyrosine kinase upon binding to the ECD. Often, the agonist will comprise a growth factor (i.e., a polypeptide that is able to stimulate cell division). Exemplary growth factors include heregulin (HRG), insulin, insulin-like growth factors I and II (IGF-I and 10 IGF-II), epidermal growth factor (EGF), interleukins (e.g., IL-8), macrophage colony-stimulating factor (M-CSF), erythropoietin (EPO), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factors alpha and beta (TGF- $\alpha$  and TGF- $\beta$ ), hepatocyte growth factor (HGF), and nerve growth factor (NGF). Alternatively, the 15 agonist can be an antibody against the rPTK (see, e.g., Yarden, Proc. Natl. Acad. Sci. USA 87:2569-2573 [1990]). However, other non-protein agonists such as small organic molecules are also encompassed by the invention.

By "antagonist" is meant a molecule which blocks agonist action. Usually, the antagonist will either: (a) bind to the rPTK and thereby 20 block binding and/or activation of the rPTK by an agonist thereto (the antagonist may bind to the ECD of the rPTK, but this is not necessarily the case) or (b) bind to the agonist and thus prevent activation of the rPTK by the agonist. This assay facilitates the detection of both types of antagonist. The antagonist may, for example, comprise a peptide fragment 25 comprising the receptor binding domain of the endogenous agonist ligand for the receptor. The antagonist may also be an antibody which is directed against the ECD of the rPTK, or against a known agonist for the rPTK. However, other non-protein molecules are also encompassed by this term.

The term "antibody" is used in the broadest sense and specifically 30 covers monoclonal antibodies and antibody compositions with polyepitopic specificity (i.e. polyclonal antibodies). The polyclonal antibodies are preferably "affinity purified" antibodies. The term "affinity purified" means that the antibodies have been purified using the antigen (e.g. the rPTK or fragment thereof or the flag polypeptide) to selectively purify the 35 polyclonal antibodies. Affinity purification can be achieved by immobilizing the antigen on an affinity column (e.g. an agarose column) and passing the polyclonal antibodies through the column. The affinity purified antibodies can be subsequently eluted from the column by changing

the elution conditions or by adding a chaotropic agent, for example. For a review of affinity purification techniques with respect to antibodies, see Current Protocols in Immunology, Ed. Coligen et al., Wiley publishers, Vols. 1 and 2, for example.

5       The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against 10 a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

15      The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of a selected antibody with a constant domain (e.g. "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous 20 proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, F(ab')<sub>2</sub>, and Fv), so long as they exhibit the desired biological activity. [See, e.g. U.S. Patent No. 4,816,567 and Mage & Lamoyi, in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc., New 25 York (1987)].

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies 30 to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler & Milstein, Nature, 256: 495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., 35 Nature, 352: 624-628 (1991) and Marks et al., J. Mol. Biol., 222: 581-597 (1991), for example.

The term "anti-phosphotyrosine antibody" refers to a molecule, usually an antibody, which binds selectively to phosphorylated tyrosine

residues in the kinase domain of a rPTK. The antibody can be polyclonal, but is desirably a monoclonal antibody. Anti-phosphotyrosine polyclonal antibodies can be made using the techniques disclosed in White and Backer, Methods in Enzymology 201:65-67 [1991] and monoclonal anti-phosphotyrosine 5 antibodies can be obtained commercially from Upstate Biologicals, Inc. (UBI, Lake Placid, NY), for example.

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly with a molecule (such as the anti-phosphotyrosine antibody). The label may be detectable 10 by itself (e.g. radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze a chemical alteration of a substrate compound or composition which is detectable. The preferred label is an enzymatic one which catalyzes a color change of a non-radioactive color reagent.

15 By "washing" is meant exposing the solid phase to an aqueous solution (usually a buffer or cell culture media) in such a way that unbound material (e.g., non-adhering cells, non-adhering capture agent, unbound ligand, receptor, receptor construct, cell lysate, or anti-phosphotyrosine antibody) is removed therefrom. To reduce background noise, it is 20 convenient to include a detergent (e.g. Triton X) in the washing solution. Usually, the aqueous washing solution is decanted from the wells of the assay plate following washing. Conveniently, washing can be achieved using an automated washing device. Sometimes, several washing steps (e.g., between about 1 to 10 washing steps) may be required.

25 By "block buffer" is meant an aqueous, pH buffered solution containing at least one blocking compound which is able to bind to exposed surfaces of the second solid phase which are not coated with capture agent. The blocking compound is normally a protein such as bovine serum albumin (BSA), gelatin, casein or milk powder and does not cross-react with any of 30 the reagents in the assay (e.g., the anti-phosphotyrosine antibodies and detection reagents). The block buffer is generally provided at a pH between about 7 to 7.5 and suitable buffering agents include phosphate and TRIS.

By "lysis buffer" is meant an aqueous, pH buffered solution 35 comprising a solubilizing detergent, one or more protease inhibitors and at least one phosphatase inhibitor (such as sodium orthovanadate). The term "solubilizing detergent" refers to a water miscible, non-ionic detergent which lyses cell membranes of eukaryotic cells but does not

denature or activate the receptor or receptor construct. Examples of suitable non-ionic detergents include Triton-X 100, Tween 20, CHAPS and Nonidet P-40 (NP40) available from Calbiochem, La Jolla, California, for example. Many other non-ionic detergents are available in the art. Examples 5 of suitable protease inhibitors include phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin, aprotinin, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride-bestatin, chymostatin and benzamidine. Preservatives (e.g., thimerosal) and one or more compounds which maintain the isotonicity of the solution (e.g., sodium chloride [NaCl] or sucrose) 10 and a buffer (e.g., Tris or PBS) are usually also present. Generally, the pH of the lysis buffer is in the range about 7 to 7.5.

Usually, following addition of the lysis buffer to the first assay plate, the first assay plate is "gently agitated" and this expression refers to the act of physically shaking the first assay plate (normally 15 using a circular motion) at a substantially low velocity. Gentle agitation does not involve mechanically disrupting the cells (e.g. by homogenizing or centrifuging the cells). Exemplary shaking velocities are in the order of 200 to 500 rpm, preferably 300 to 400 rpm in a Bellco orbital shaker, for example.

20 II. Modes for Practicing the Invention

1. Kinase Receptor Activation - KIRA

The first stage of the assay involves phosphorylation of the kinase domain of a kinase receptor, wherein the receptor is present in the cell membrane of a eukaryotic cell. The receptor may be an endogenous receptor 25 or nucleic acid encoding the receptor may be transformed into the cell. In one embodiment of the invention, nucleic acid encoding a receptor construct is transformed into the cell. Exemplary techniques for transforming the cell with either the receptor or the receptor construct nucleic acid follow.

30 A. Transformation of the cells

The instant invention provides a substantial improvement over soluble kinase receptor assays insofar as it is considered to more accurately reflect the activity of the receptor *in situ*. It has been discovered that it is possible to transform eukaryotic cells with a receptor construct 35 (comprising the kinase receptor and either an amino- or carboxyl-terminal flag polypeptide) so that the receptor construct assembles itself appropriately in the cell membrane and still retains tyrosine kinase

activity which can be detected in the ELISA stage of the assay. This provides a generic assay for measuring tyrosine kinase activity of any tyrosine kinase of interest.

If a suitable capture agent as described herein is available for a selected rPTK, cells can be transformed with the nucleic acid encoding the receptor alone, without the flag polypeptide. Alternatively, if cells are available which produce the receptor (e.g., MCF-7 cells which produce the HER2 receptor), it is not necessary to transform the cells for use in the assay.

In order to transform the cells with the nucleic acid encoding the rPTK or receptor construct, nucleic acid encoding the rPTK and, optionally, the flag polypeptide, is isolated. This can be achieved by screening a cDNA or genomic library known to contain the DNA encoding the rPTK or flag polypeptide of interest with a selected labelled probe (e.g., an antibody or oligonucleotide- probe) for the rPTK or flag polypeptide, using standard procedures as described in chapters 10-12 of Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989), for example. Alternatively, the nucleic acid encoding the flag polypeptide can be made synthetically using an oligo-synthesizing machine (Applied Biosystems, CA). An alternative means to isolate the nucleic acid encoding the rPTK or flag polypeptide is to use PCR methodology as described in section 14 of Sambrook et al., *supra*. Isolation of only the ECD of the rPTK of interest is required, since this nucleic acid can be fused to the nucleic acid encoding the TM and ICD of the Rse-flag polypeptide construct disclosed herein. See Figures 1A - 1C and SEQ ID NOS: 1 and 2. If necessary however, conventional primer extension procedures as described in section 7.79 of Sambrook et al., *supra*, can be used to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

A preferred method of practicing this invention is to use carefully selected oligonucleotide sequences to screen cDNA libraries from various tissues, preferably mammalian cell lines having the rPTK of interest. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized.

The oligonucleotide must be labeled such that it can be detected upon hybridization to DNA in the library being screened. The preferred method of labeling is to use  $^{32}\text{P}$ - labeled ATP with polynucleotide kinase, as is well known in the art, to radiolabel the oligonucleotide. However, other

methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labeling.

In order to provide nucleic acid encoding a receptor construct, nucleic acid encoding the rPTK is fused at its 3' end to nucleic acid 5 encoding the N-terminus of the flag polypeptide. Alternatively, the nucleic acid encoding the rPTK will be fused at its 5' end to nucleic acid encoding the carboxyl terminus of the flag polypeptide. Thus, the flag polypeptide is provided at either the carboxyl- or amino- terminus of the receptor construct. Examples of suitable flag polypeptides are provided above. 10 Selection of other suitable flag polypeptides is possible using the techniques described herein.

In order to generate fusions between the Rse.flag reagent and a rPTK of interest, the nucleic acid encoding the ECD of the rPTK of interest is fused at its 3' end to the nucleic acid encoding the amino terminus of the 15 Rse.flag reagent.

The nucleic acid (e.g., cDNA or genomic DNA) encoding the rPTK or receptor construct is then inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available to the skilled practitioner but must be compatible with the cell 20 which is to be used in the assay. The vector will have vector components the presence of which will depend on various factors. Such components include, for example, a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Selection of these vector components shall be 25 described below.

Incorporation of a signal sequence into the expression vector is required since the rPTK or receptor construct must be transported to the cell membrane where it is positioned such that the ECD faces the external milieu of the cell. Therefore, a signal sequence suitable for positioning 30 the rPTK or receptor construct in such a manner is used. The signal sequence is generally a component of the vector, or it may be a part of the rPTK or receptor construct DNA that is inserted into the vector. If a heterologous signal sequence is used, it is from those that are recognized and processed (i.e., cleaved by a signal peptidase) by the host cell.

35 For yeast secretion the native signal sequence may be substituted by, e.g., the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces*  $\alpha$ -factor leaders, the latter described in U.S. Patent No. 5,010,182 issued 23 April 1991), or acid phosphatase

leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cells expression of the DNA encoding the native signal sequence (e.g., the rPTK pre-sequence that normally directs secretion of 5 rPTK from mammalian cells *in vivo*) is satisfactory, although other mammalian signal sequences may be suitable, such as signal sequences from other animal rPTKs, and signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex gD signal.

10 The DNA for such precursor region is ligated in reading frame to DNA encoding the rPTK or receptor construct.

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector 15 to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. The 2 $\mu$  plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not 20 needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organisms but can be transformed into another organism for expression. For example, a vector is cloned in 25 *E. coli* and then the same vector is transformed into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by 30 including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transformation of *Bacillus* with this vector results in homologous recombination with the genome and insertion of rPTK or receptor construct DNA. However, the recovery of genomic DNA encoding the rPTK or receptor construct is more complex than that of an exogenously 35 replicated vector because restriction enzyme digestion is required to excise the rPTK or receptor construct DNA.

Expression and cloning vectors usually contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the

survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other 5 toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a 10 heterologous gene express the DNA encoding a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern et al., J. Molec. Appl. Genet. 1:327 [1982]), mycophenolic acid (Mulligan et al., Science 209:1422 [1980]) or hygromycin (Sugden et al., Mol. Cell. Biol. 5:410-413 [1985]). 15 The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the 20 rPTK or receptor construct nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection 25 agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes the rPTK or receptor construct. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of 30 recombinant cells. Increased quantities of rPTK or receptor construct are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, etc.

For example, cells transformed with the DHFR selection gene are first 35 identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and

## 26

propagated as described by Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding the rPTK or receptor construct. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

10 Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding the rPTK or receptor construct, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the 15 selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

20 A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 (Stinchcomb et al., Nature 282:39 [1979]; Kingsman et al., Gene 7:141 [1979]; or Tschemper et al., Gene 10:157 [1980]). The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, Genetics 85:12 [1977]). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. 25 Similarly, Leu2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

30 In addition, vectors derived from the 1.6  $\mu$ m circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts. Bianchi et al., Curr. Genet. 12:185 (1987). More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis*. Van den Berg, Bio/Technology 8:135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed. Fleer et al., Bio/Technology 9:968-975 (1991).

35 Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the rPTK or receptor construct nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally

within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the rPTK nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are 5 promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to rPTK or receptor 10 construct-encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native rPTK promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the rPTK or receptor construct DNA. The promoter will be one 15 which results in the accumulation of suitable numbers of receptor or receptor construct in the cell membrane of the transformed cell (i.e. so that autophosphorylation of the receptor is detectable in the ELISA but constitutive phosphorylation does not occur). Selection of a suitable promoter to achieve this is possible following the guidelines herein for 20 selecting cells for use in the KIRA ELISA.

Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start 25 of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts 30 include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem. 255:2073 [1980]) or other glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg., 7:149 [1968]; and Holland, Biochemistry 17:4900 [1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 35 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are

the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and 5 promoters for use in yeast expression are further described in Hitzeman et al., EP 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

rPTK or receptor construct transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the 10 genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin 15 promoter, from heat-shock promoters, and from the promoter normally associated with the rPTK or receptor construct sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral 20 origin of replication. Fiers et al., Nature 273:113 (1978); Mulligan and Berg, Science 209:1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA 78:7398-7402 (1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway et al., Gene 18:355-360 (1982). A system for 25 expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. 4,419,446. A modification of this system is described in U.S. 4,601,978. See also Gray et al., Nature 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes et al., Nature 297:598-601 (1982) on expression of human  $\beta$ -interferon cDNA 30 in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79:5166-5170 (1982) on expression of the human interferon  $\beta$ 1 gene in cultured mouse and rabbit cells; and Gorman et al., Proc. Natl. Acad. Sci. USA 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney 35 cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

Transcription of DNA encoding the rPTK or receptor construct by higher eukaryotes may be increased, if increased numbers of the rPTK or receptor construct per cell are required to facilitate detection in the ELISA stage of the assay. This may be achieved by inserting an enhancer 5 sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' (Laimins et al., Proc. Natl. Acad. Sci. USA 78:993 [1981]) and 3' (Lusky et al., Mol. Cell Bio. 3:1108 [1983]) to 10 the transcription unit, within an intron (Banerji et al., Cell 33:729 [1983]), as well as within the coding sequence itself (Osborne et al., Mol. Cell Bio. 4:1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin,  $\alpha$ -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. 15 Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into 20 the vector at a position 5' or 3' to the rPTK or receptor construct-encoding sequence, but is preferably located at a site 5' from the promoter.

Expression vectors used in eukaryotic host cells will also contain sequences necessary for the termination of transcription and for 25 stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the rPTK or receptor construct.

30 Construction of suitable vectors containing one or more of the above listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, 35 the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced

by the method of Messing et al., Nucleic Acids Res. 9:309 (1981) or by the method of Maxam et al., Methods in Enzymology 65:499 (1980).

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the rPTK or receptor construct in recombinant vertebrate cell culture are described in Gething et al., Nature 293:620-625 (1981); Mantei et al., Nature 281:40-46 (1979); Levinson et al.; EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of rPTK or receptor construct DNA is pRK5 (EP 307,247) or pSVI6B (PCT pub. no. WO 91/08291 published 13 June 1991).

Examples of suitable eukaryotic cell lines for transformation include Saccharomyces cerevisiae, Schizosaccharomyces pombe (Beach and Nurse, Nature 290:140 [1981]; EP 139,383 published 2 May 1985); Kluyveromyces hosts (U.S. 4,943,529; Fleer et al., Bio/Technology 9:968-975 [1991]) and Aspergillus hosts such as A. nidulans (Ballance et al., Biochem. Biophys. Res. Commun. 112:284-289 [1983]; Tilburn et al., Gene 26:205-221 [1983]; Yelton et al., Proc. Natl. Acad. Sci. USA 81:1470-1474 [1984]) and A. niger (Kelly and Hynes, EMBO J. 4:475-479 [1985]), among lower eukaryotic host microorganisms.

Examples of useful animal host cell lines for transformation include monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen. Virol. 36:59 [1977]); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216 [1980]); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23:243-251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (WI38, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci. 383:44-68 [1982]); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or as a chromosomal

integrand. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. Successful transformation is generally recognized when any indication of the operation of this vector occurs within the host cell.

5 For mammalian cells, the calcium phosphate precipitation method of Graham and Van der Eb, Virology 52:456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. 4,399,216 issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van Solingen et  
10 al., J. Bact. 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA) 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, etc., may also be used. For various techniques for transforming mammalian  
15 cells, see Keown et al., Methods in Enzymology (1989), Keown et al., Methods in Enzymology 185:527-537 (1990), and Mansour et al., Nature 336:348-352 (1988).

The mammalian host cells used to produce the rPTK or receptor construct may be cultured in a variety of media. Commercially available  
20 media such as Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, Meth. Enz. 58:44 (1979), Barnes and Sato, Anal. Biochem. 102:255 (1980), U.S. 4,767,704; 4,657,866; 4,927,762; or  
25 4,560,655; WO 90/03430; WO 87/00195; U.S. Patent Re. 30,985; or U.S. Patent No. 5,122,469, the disclosures of each of which are incorporated herein by reference, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts  
30 (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements  
35 may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed., IRL Press, 1991.

5 Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas, Proc. Natl. Acad. Sci. USA 77:5201-5205 [1980]), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences  
10 provided herein. Various labels may be employed, most commonly radioisotopes, particularly  $^{32}\text{P}$ . However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as  
15 radionuclides, fluorophores, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the  
20 formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining to quantitate directly the expression of gene product.

25 **B. Selecting cells for use in the assay**

As mentioned above, the cells to be subjected to the assay can be (a) cells having an endogenous receptor, (b) cells which have been transformed with a rPTK, or (c) cells transformed with a receptor construct. The suitability of the cells for use in the assay is investigated.

30 Cells having the endogenous rPTK can be subjected to a test-run KIRA ELISA using a known ligand to the PTK (e.g. an agonist antibody) and a control (e.g. the diluent for the agonist antibody). A range of ligand concentrations such as those used herein (see Examples 1, 2 and 3) will be used to determine whether sufficient numbers of the receptor are present  
35 in the cells being tested. In order to discover whether a cell line is unsuitable because the receptor is constitutively phosphorylated, the cell line can be subjected to the KIRA ELISA disclosed herein, wherein it is exposed to both positive and negative controls (e.g. a known agonist ligand

in cell culture media as described herein as a positive control and the cell culture media without the agonist ligand as the negative control). If phosphorylation of the receptor is detected for both positive and negative controls, this may be indicative that constitutive phosphorylation 5 of the receptor is occurring. However, it is possible that a constituent of the serum in the cell culture media is activating the receptor. Thus, the cells can be "starved" in serum-free media for about 2-12 hours (depending on cell survival) and then the assay is repeated using the positive and negative controls. If activation is detected for both 10 controls, the cell line may be considered unsuitable and another cell line can be tested.

If the cell line is transformed with the receptor (without the flag polypeptide) a strategy similar to that depicted in Figure 4 can be used to discover whether or not the cell line is suitable for use in the assay. 15 As a first step, successful transformation and expression of the nucleic acid encoding the rPTK is determined (see Figure 4, step b). In order to identify whether the ECD of the rPTK is present on the surface of the cells, flow cytometric analysis can be performed using an antibody to the ECD of the receptor. The antibody can be made using the techniques for 20 generating antibodies discussed herein. Flow cytometric analysis can be carried out using the techniques described in Current Protocols in Immunology, Ed. Coligen et al., Wiley publishers, Vols. 1 and 2, for example. Briefly, flow cytometric analysis involves incubating intact cells (having the receptor) with antibodies to the ECD thereof, followed 25 by washing. The antibody-bound cells are then incubated with species specific anti-antibody conjugated to a fluorochrome. Following washing, the labeled cells are analyzed by fluorescence-activated flow cytometry to detect whether the ECD is present on the surface of the cells.

In the following step, i.e. Figure 4, step (c), the ability of the 30 cell-bound receptor to be activated is tested. In order to determine this, the transformed cells are exposed to a known agonist to the receptor (e.g. the endogenous ligand or an agonist antibody for the receptor). Following exposure, the cells are lysed in a suitable buffer (e.g. sodium dodecylbenzenesulfonate in phosphate buffered saline; SDS in PBS) and 35 subjected to Western blotting with anti-phosphotyrosine antibodies as described in Wang, Molecular and Cellular Biology 5(12):3640-3643 (1985); Glenney et al., Journal of Immunological Methods 109:277-285 (1988); Kamps, Methods in Enzymology 201:101-110 (1991); Kozma et al., Methods in

Enzymology 201:28-43 (1991); Holmes et al., Science 256:1205-10 (1992); or Corfas et al., PNAS, USA 90:1624-1628 (1993), for example.

Assuming the Western blotting step indicates that the rPTK can be activated, a KIRA ELISA test run can be performed, see Figure 4 step (d),  
5 to further establish whether or not the transformed cell line can be used  
in the assay.

In the preferred embodiment of the invention, the KIRA ELISA is a "generic" assay insofar as any rPTK of interest can be studied regardless  
10 of the availability of receptor-specific reagents (i.e., capture agent).  
This embodiment employs a receptor construct having a flag polypeptide at  
either the amino or carboxyl terminus of the receptor.

If the flag polypeptide is provided at the NH<sub>2</sub>-terminus (see, e.g.,  
the gD.trk A, B and C receptor constructs disclosed in Example 3), the  
procedure for selecting a transformed cell line for use in the assay  
15 summarized in Figure 4 can be performed. In this embodiment, the cells are  
transformed with the flag polypeptide-receptor construct as described  
earlier herein. See step (a). In step (b), successful transformation of the  
receptor and flag polypeptide (i.e. the receptor construct) is confirmed.  
In order to study this, two-dimensional flow cytometric analysis can be  
20 performed using antibodies to both the flag polypeptide and the ECD of the  
receptor. Techniques for two-dimensional flow cytometric analysis are  
disclosed in Current Protocols in Immunology, supra. Assuming successful  
transformation of the receptor construct is demonstrated, steps (c) and (d)  
25 of Figure 4 are then performed. See the discussion above, for an  
explanation of steps (c) to (d) of Figure 4.

A technique for identification of cells which have been successfully  
transformed with the receptor construct having a C-terminal flag  
polypeptide and which cells are also suitable for use in the assay is  
illustrated in Figure 5. Following cell transformation [step (a)],  
30 successful transformation of the receptor is determined by flow cytometric  
analysis using an antibody directed against the ECD of the receptor of  
interest, for example. Flow cytometric analysis can be performed  
substantially as described above. This forms step (b) of the procedure  
outlined in Figure 5.

Following step (b), successful transformation of the entire receptor  
construct (including the COOH-terminal flag polypeptide) is analyzed in  
step (c). This can be achieved by lysing the cells (using techniques for  
lysing cells disclosed herein) and immunoprecipitating the membrane extract

with an antibody against the receptor of interest. This immunoprecipitated membrane extract is then subjected to Western blot analysis with antibodies specific for the flag polypeptide. Alternatively, rPTK-specific ELISA analysis of anti-flag polypeptide captured membrane lysate can be carried 5 out. Briefly, this involves coating ELISA wells with appropriate flag specific capture agent. The wells are blocked, washed, and the lysate is then incubated in the wells. Unbound receptor construct is removed by washing. The wells are then reacted with receptor-specific antibody or antibodies, either directly or indirectly conjugated to HRPO. The wells 10 are washed and the HRPO is then exposed to the chromogenic substrate (e.g., TMB).

Steps (d) and (e), i.e., detecting receptor activation and KIRA ELISA test run, are essentially the same as those steps described above.

Once useful cells are identified, they are subjected to the KIRA 15 stage of the instantly claimed assay.

C. Coating the first solid phase with the cells

The first solid phase (e.g. a well of a first assay plate) is coated with cells having the endogenous receptor or cells which have been transformed pursuant to the preceding sections.

20 Preferably, an adherent cell line is chosen, so that the cells naturally adhere to the first solid phase. However, use of an adherent cell line is not essential. For example, non-adherent cells (e.g. red blood cells) can be added to round bottomed wells of an assay plate such as that sold by Becton Dickinson Labware, Lincoln Park, New Jersey, for example. 25 The assay plate is then placed in a plate carrier and centrifuged so as to create a pellet of cells adhering to the base of the wells. The cell culture supernatants are removed using a pipette. Thus, use of an adherent cell is clearly advantageous over non-adherent cells since it reduces variability in the assay (i.e, the cells in the pellet of the round bottom 30 wells may be taken up with the supernatant when the alternative method is used).

The cells to be added to the wells of the first assay plate may be maintained in tissue culture flasks and utilized when cells densities of about 70-90% confluence are achieved. Then, generally between about 1 x 35  $10^4$  to  $3 \times 10^5$  (and preferably  $5 \times 10^4$  to  $1 \times 10^5$ ) cells are seeded per flat-bottom well, using a pipette, for example. It has been found that, contrary to expectations, addition of cell concentrations mentioned above is sufficient to enable activation of the rPTK to be measured in the ELISA

stage of the assay, without the need to concentrate or clarify the cells or cell lysate prior thereto. Often, the cells are diluted in culture medium prior to seeding them in the wells of the microtiter plate to achieve the desired cell densities.

5        Usually, the cells are cultured in the microtiter plates for a sufficient period of time to optimize adherence to the wells thereof, but not too long such that the cells begin to deteriorate. Thus, incubation for about 8 to 16 hours at a temperature which is the physiological optimum for the cells (usually about 37°C) is preferred. Suitable media for  
10      culturing the cells are described in Section 1A above. Culturing in 5% CO<sub>2</sub> is recommended.

Following incubation overnight, the well supernatants are decanted and excess supernatant may be further removed by lightly tamping the microtiter plates with an absorbent substrate, e.g., a paper towel, but a  
15      sponge works equally well. Thus, a substantially homogeneous layer of adhering cells remains on the internal surfaces of the individual wells of the microtiter plate. These adhering cells are then exposed to the analyte.

20      *D. Preparation and addition of the analyte*

As mentioned above, the analyte may comprise an agonist ligand (or suspected agonist) or an antagonist (or suspected antagonist) for the rPTK of interest. The ligand may be an endogenous polypeptide, or a synthetic molecule, such as an inorganic or organic molecule. Usually, the ligand is a polypeptide. This assay is useful for screening molecules which activate  
25      (or antagonize activation) of the tyrosine kinase receptor of interest. Thus, the assay can be used for developing therapeutically effective molecules.

Where the ligand is an agonist, the molecule can comprise the native growth factor e.g., heregulin (HRG), insulin, insulin-like growth factors  
30      I and II (IGF-I and IGF-II), epidermal growth factor (EGF), interleukins (e.g., IL-8), macrophage colony-stimulating factor (M-CSF), erythropoietin (EPO), platelet-derived growth factor (PDGF), transforming growth factors alpha and beta (TGF- $\alpha$  and TGF- $\beta$ ), hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and nerve growth factor (NGF). Many of these  
35      growth factors are available commercially. Alternatively, the growth factor can be made by peptide synthesis or recombinant techniques which are described herein. Synthetic small molecule agonists can similarly be

generated by those skilled in the art using conventional chemical synthesis techniques.

Where the ligand is present in a biological fluid, the analyte can be prepared using techniques which are well known in the art. Body fluid such as blood or amniotic fluid may be used directly, however concentration may be required. If the analyte to be tested comprises a particular tissue, the cells thereof can be grown in cell culture and the supernatant can be tested for secreted ligand.

Often, the ligand is diluted in an aqueous diluent (such as cell culture media) so that a standard curve can be generated. However, the ligand may be present in a cell or a cell component (e.g., the cell membrane). In particular, it has been found that the assay can be used to detect the presence of a ligand in the cell membrane of a selected cell line. This is clearly useful for discovering a novel endogenous ligand for a known rPTK.

The ligand composition is added to each well which contains the adhering cells using a pipette, for example. At least one control well (e.g. to which the aqueous diluent for the ligand is added) is included in the assay.

20 The adhering cells are usually stimulated for a sufficient period of time to optimize the signal, but not too long such that the signal decreases as a consequence of dephosphorylation of the rPTK by endogenous phosphatases. A suitable stimulation period is between about 10 to 60 minutes, preferably about 30 minutes at a physiologically optimal 25 temperature for the cells (usually about 37°C).

Following activation, well supernatants are decanted and the plates can then be lightly tamped with an absorbent substrate to remove excess supernatant.

30 The assay can be used to detect antagonist ligands for the rPTK of interest. Antagonists generally fall into two categories (a) ones which bind to the rPTK and thereby block binding and/or activation of the rPTK by an agonist thereto (the antagonist may bind to the ECD, but this is not necessarily the case) and (b) those which bind to the agonist and thus prevent activation of the rPTK by the agonist.

35 In order to detect antagonist molecules from category (a) above, the cells are exposed to the suspected antagonist ligand substantially as mentioned above. Following exposure to the antagonist, the well supernatants are decanted and the plates are lightly tamped. Then, a known

agonist (e.g., the endogenous growth factor) is added to the washed cells essentially as discussed in the preceding paragraphs, following which, the well supernatants are decanted and plates are lightly tamped. Alternatively, a composition comprising both the antagonist and agonist can 5 be added to the adhering cells substantially as discussed above. Ability of the suspected antagonist to block binding and/or activation of the rPTK can subsequently be measured by ELISA as discussed below.

To detect antagonist molecules from category (b) above, a known agonist is pre-incubated with the suspected antagonist prior to the KIRA 10 stage of the assay. This incubation is carried out for a sufficient period of time to enable a complex of the antagonist-agonist to form; from 30 min. to 12 hours, for example. This complex is then subjected to the assay with the non-complexed agonist and antagonist used as controls.

Following exposure to the agonist (and optionally the antagonist) 15 ligand, the cells are lysed, as discussed below.

#### E. Solubilizing the cells

In this step of the assay, the cells are lysed so as to solubilize the rPTK such that it remains activated (i.e., the tyrosine residues remain phosphorylated) for the ELISA stage of the assay. Thus, the cells are lysed 20 using a lysis buffer as described above which serves to solubilize the rPTK or receptor construct, yet does not dephosphorylate or denature the rPTK.

Where microtiter plates are used as mentioned above, about 75 to 200 $\mu$ l of lysis buffer is added to each well. The plates can then be agitated gently using a plate shaker (e.g., such as that sold by Bellco 25 Instruments, Vineland, NJ) for about 1 to 2 hours. Shaking can be carried out at room temperature.

#### 2. Enzyme-Linked Immunosorbent Assay - ELISA

The second stage of the assay involves a sandwich ELISA performed in the second assay plate. In order to carry out the ELISA, a capture agent 30 is prepared.

##### A. Preparation of the capture agent

As mentioned above, the capture agent often comprises a polyclonal antibody (usually an affinity purified polyclonal antibody) or monoclonal antibody. Other capture agents are envisaged and are discussed in the 35 definitions section above. The capture agent either binds specifically to the kinase receptor, or to the flag polypeptide (i.e. the antigen).

Polyclonal antibodies to the antigen (either the receptor or the flag polypeptide) generally are raised in animals by multiple subcutaneous (sc)

or intraperitoneal (ip) injections of the antigen or an antigenic fragment thereof (often the ECD of the rPTK) and an adjuvant. It may be useful to conjugate the antigen or a fragment containing the target amino acid sequence to a protein that is immunogenic in the species to be immunized 5 (e.g., keyhole limpet hemocyanin), using a bifunctional or derivatizing agent.

The route and schedule for administration of immunogen to the host animal or cultured antibody-producing cells therefrom are generally in keeping with established and conventional techniques for antibody 10 stimulation and production. While mice are frequently employed as the test model, it is contemplated that any mammalian subject including human subjects or antibody-producing cells obtained therefrom can be manipulated according to the processes of this invention to serve as the basis for production of mammalian, including human, hybrid cell lines.

15 Animals are typically immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1  $\mu$ g of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freund's 20 complete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. 7 to 14 days later animals are bled and the serum is assayed for anti-antigen titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same 25 antigen, but conjugated to a different protein and/or through a different cross-linking agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

After immunization, monoclonal antibodies can be prepared by recovering immune cells (typically spleen cells or lymphocytes from lymph 30 node tissue) from immunized animals and immortalizing the cells in conventional fashion, e.g., by fusion with myeloma cells or by Epstein-Barr (EB)-virus transformation and screening for clones producing the desired antibody. The hybridoma technique described originally by Kohler and Milstein, Eur. J. Immunol. 6:511 (1976), and also described by Hammerling 35 et al., In: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981) has been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens.

It is possible to fuse cells of one species with another. However, it is preferable that the source of the immunized antibody producing cells and the myeloma be from the same species.

The hybrid cell lines can be maintained in culture in cell culture media. The cell lines of this invention can be selected and/or maintained in a composition comprising the continuous cell line in hypoxanthine-aminopterin-thymidine (HAT) medium. In fact, once the hybridoma cell line is established, it can be maintained on a variety of nutritionally adequate media. Moreover, the hybrid cell lines can be stored and preserved in any number of conventional ways, including freezing and storage under liquid nitrogen. Frozen cell lines can be revived and cultured indefinitely with resumed synthesis and secretion of monoclonal antibody.

The secreted antibody is recovered from tissue culture supernatant by conventional methods such as precipitation, ion exchange chromatography, affinity chromatography, or the like. The antibodies described herein are also recovered from hybridoma cell cultures by conventional methods for purification of IgG or IgM, as the case may be, that heretofore have been used to purify these immunoglobulins from pooled plasma, e.g., ethanol or polyethylene glycol precipitation procedures. The purified antibodies are then sterile filtered. Where the antibody is a polyclonal antibody, it is generally affinity purified using an affinity column generated from the antigen of interest so as to provide a substantially specific capture antibody. Affinity chromatography is usually preceded by other purification techniques, such as liquid chromatography.

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated via the techniques described in McCafferty et al., Nature, 348:552-554 (1990), using the flag polypeptide, rPTK, or a fragment thereof, to select for a suitable antibody or antibody fragment. Clackson et al., Nature, 352:624-628 (1991) and Marks et al., J. Mol. Biol., 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Mark et al., Bio/Technol., 10:779-783 [1992]), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse et al., Nuc. Acids Res., 21:2265-2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for

isolation of "monoclonal" antibodies which are encompassed by the present invention.

DNA encoding the monoclonal antibodies of the invention is readily isolated and sequenced using conventional procedures (e.g., by using 5 oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary 10 (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences, Morrison et al., Proc. 15 Nat. Acad. Sci. 81, 6851 (1984), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In that manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity of an anti-rPTK or anti-flag polypeptide monoclonal antibody herein. Thus, the antibody may 20 be made by recombinant DNA methods (Cabilly et al., U.S. Pat. No. 4,816,567).

Binding of the capture agent is not affected by the presence or absence of a ligand bound to the receptor and the capture agent does not sterically block access to the phosphorylated tyrosine(s) by the anti- 25 phosphotyrosine antibody. Furthermore, the capture agent does not, of course, activate the receptor of interest. In order to screen for an antibody having these characteristics, the procedure outlined in Figure 3 can be carried out.

First, once the capture agent (e.g. an antibody or streptavidin) has 30 been chosen, binding to either the receptor or the flag polypeptide (where a receptor construct is to be used in the assay) is confirmed. This can be determined by flow cytometric analysis, immuno-precipitation or antigen-coat ELISA, for example. Flow cytometric analysis has been described above. Immunoprecipitation usually involves lysing the cells (having the 35 receptor or receptor construct) in non-ionic detergent (e.g. 0.5% Triton X-100) in a suitable buffer (e.g. PBS) and the cell lysates thus obtained are then incubated with the potential anti-receptor or anti-flag polypeptide capture agent. The immune complexes are precipitated with

either (a) anti-capture agent antibodies in the presence of polyethylene glycol (PEG) which enhances precipitation of the immune complex or with (b) insoluble (e.g. agarose bound) protein A or protein G. The immunoprecipitated material is then analyzed by polyacrylamide gel 5 electrophoresis (PAGE). For antigen-coat ELISA, ELISA wells are coated overnight with either the purified receptor, purified flag polypeptide or purified receptor construct. The coated wells are then exposed to the potential capture agent and screened with HRPO-conjugated species specific anti-capture agent antibody.

10 The ability of the capture agent to bind to the receptor or flag polypeptide in the presence of a ligand to the receptor is also confirmed. This can be analyzed by incubating the receptor or receptor construct with a known ligand for the receptor (e.g. the endogenous growth factor or an agonist antibody thereto). Flow cytometric analysis, immunoprecipitation 15 or antigen-coat ELISA can then be performed substantially as described above to investigate binding of the capture agent.

Assuming the capture agent is suitable as determined by the preceding two steps, it is then shown that the capture agent does not induce receptor activation (i.e. autophosphorylation) either before or after cell lysis. 20 Thus, the cell-bound receptor or receptor construct is exposed to either the potential capture agent or a negative control (e.g. a control antibody which does not activate the receptor). Following cell lysis, the receptor or receptor construct can be subjected to Western blot analysis using labeled anti-phosphotyrosine antibodies. See, e.g., Glenney et al., 25 Journal of Immunological Methods 109:277-285 (1988); Kamps, Methods in Enzymology 201:101-110 (1991); Kozma et al., Methods in Enzymology 201:28-43 (1991); or Holmes et al., Science 256:1205-10 (1992). To establish whether the capture agent induces receptor activation following cell lysis, a trial run of the KIRA ELISA (with both the capture agent and a negative 30 control as discussed above) can be performed.

Finally, the ability of an anti-phosphotyrosine antibody (e.g. biotinylated anti-phosphotyrosine antibody) to bind the activated receptor in the presence of the potential capture agent is confirmed by a trial run in the KIRA ELISA disclosed herein.

35 Assuming the capture agent meets all the criteria specified above, it has good potential for use in the KIRA ELISA.

Once a suitable capture agent has been prepared, the second solid phase is coated therewith. Between about 0.1 to 10 µg/ml of capture agent

can be added to each well of the second assay plate using a pipette, for example. The capture agent is often provided in a buffer at a high pH (e.g., between about 7.5 to 9.6) so that it has an increased overall charge and therefore displays enhanced binding to the second assay plate. Usually, 5 the capture agent will be incubated in the wells for between about 8 to 72 hours to enable a sufficient coating of the capture agent to form on the inside walls of the wells. This incubation is generally carried out at low temperatures (e.g., between about 3-8°C) to avoid or reduce degradation of the capture agent.

10 Following incubation, the wells of the plate are decanted and tamped lightly with an absorbent substrate. Non-specific binding is then blocked. In order to achieve this, a block buffer, is added to the wells. For example, a block buffer containing bovine serum albumin (BSA) such as that sold by Intergen Company, Purchase, NY, is suitable. It has been found that 15 addition of between about 100 to 200 $\mu$ l of block buffer to each well followed by gentle agitation at room temperature for between about 1-2 hours is sufficient to block non-specific binding. It is also possible to add the block buffer directly to the cell lysate obtained in the previous step rather than to the second assay plate.

20 Following this, the capture agent-coated plates are washed several times (usually between about 3-8 times) with a wash buffer. The wash buffer can comprise phosphate buffered saline (PBS) at pH 7.0 to 7.5, for example. However, other wash buffers are available which can also be used. Conveniently, an automated plate washer, such as the ScanWasher 300 25 (Skatron Instruments, Inc., Sterling, VA) can be used for this, and other, washing steps of the assay.

#### B. Measuring tyrosine phosphorylation

The activated, solubilized rPTK (or receptor construct) is then added to the wells having the capture agent adhering thereto. As a general 30 proposition, about 80% of cell lysate obtained as mentioned under Section 1E above can be added to each well (i.e., about 60 to 160  $\mu$ l depending on the original volume of the wells). The lysate is incubated with the capture agent for an adequate period of time to enable the rPTK to be captured in the wells, e.g., from 1 to 3 hours. Incubation can be carried 35 out at room temperature.

Unbound cell lysate is then removed by washing with wash buffer. Following this washing step, an amount of the anti-phosphotyrosine antibody which is equal to, or less than, the amount of block buffer added

previously, is added to each well. For example, about 50 to 200 $\mu$ l of an anti-phosphotyrosine antibody preparation having between about 0.3 to 0.5  $\mu$ g/ml of antibody in a suitable buffer (e.g., PBS with a detergent such as those included in the lysis buffer) is added to the well. This is followed by a washing step to remove unbound anti-phosphotyrosine antibody.

Tyrosine phosphorylation is then quantified by the amount of anti-phosphotyrosine antibody binding to the second solid phase. Many systems for detecting the presence of an antibody are available to those skilled in the art. Some examples follow.

Generally, the anti-phosphotyrosine antibody will be labelled either directly or indirectly with a detectable label. Numerous labels are available which can be generally grouped into the following categories:

(a) Radioisotopes, such as  $^{35}\text{S}$ ,  $^{14}\text{C}$ ,  $^{125}\text{I}$ ,  $^3\text{H}$ , and  $^{131}\text{I}$ . The antibody can be labeled with the radioisotope using the techniques described in Current Protocols in Immunology, *supra*, for example and radioactivity can be measured using scintillation counting.

(b) Fluorescent labels such as rare earth chelates (europium chelates) or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, Lissamine, phycoerythrin and Texas Red are available. The fluorescent labels can be conjugated to the antibody using the techniques disclosed in Current Protocols in Immunology, *supra*, for example. Fluorescence can be quantified using a fluorimeter (Dynatech).

(c) Various enzyme-substrate labels are available and U.S. Patent No. 4,275,149 provides a review of some of these. The enzyme generally catalyses a chemical alteration of the chromogenic substrate which can be measured using various techniques. For example, the enzyme may catalyze a color change in a substrate, which can be measured spectrophotometrically. Alternatively, the enzyme may alter the fluorescence or chemiluminescence of the substrate. Techniques for quantifying a change in fluorescence are described above. The chemiluminescent substrate becomes electronically excited by a chemical reaction and may then emit light which can be measured (using a Dynatech ML3000 chemiluminometer, for example) or donates energy to a fluorescent acceptor. Examples of enzymatic labels include luciferases (e.g., firefly luciferase and bacterial luciferase; U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, malate dehydrogenase, urease, peroxidase such as horseradish peroxidase (HRPO), alkaline phosphatase,  $\beta$ -galactosidase, glucoamylase, lysozyme, saccharide oxidases (e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate

dehydrogenase), heterocyclic oxidases (such as uricase and xanthine oxidase), lactoperoxidase, microperoxidase, and the like. Techniques for conjugating enzymes to antibodies are described in O'Sullivan et al., Methods for the Preparation of Enzyme-Antibody Conjugates for use in Enzyme Immunoassay, in Methods in Enzym. (ed J. Langone & H. Van Vunakis), Academic press, New York, 73: 147-166 (1981) and Current Protocols in Immunology, supra.

Examples of enzyme-substrate combinations include, for example:

(i) Horseradish peroxidase (HRPO) with hydrogen peroxidase as a substrate, wherein the hydrogen peroxidase oxidizes a dye precursor (e.g. orthophenylene diamine [OPD] or 3,3',5,5'-tetramethyl benzidine hydrochloride [TMB]).

(ii) alkaline phosphatase (AP) with para-Nitrophenyl phosphate as chromogenic substrate.

(iii)  $\beta$ -D-galactosidase ( $\beta$ -D-Gal) with a chromogenic substrate (e.g. p-nitrophenyl- $\beta$ -D-galactosidase) or fluorogenic substrate 4-methylumbelliferyl- $\beta$ -D-galactosidase.

Numerous other enzyme-substrate combinations are available to those skilled in the art. For a general review of these, see U.S. Patent Nos. 4,275,149 and 4,318,980.

Sometimes, the label is indirectly conjugated with the antibody. The skilled artisan will be aware of various techniques for achieving this. For example, the antibody can be conjugated with biotin and any of the three broad categories of labels mentioned above can be conjugated with avidin, or vice versa. Biotin binds selectively to avidin and thus, the label can be conjugated with the antibody in this indirect manner. See, Current Protocols in Immunology, supra, for a review of techniques involving biotin-avidin conjugation. Alternatively, to achieve indirect conjugation of the label with the antibody, the antibody is conjugated with a small hapten (e.g. digoxin) and one of the different types of labels mentioned above is conjugated with an anti-hapten antibody (e.g. anti-digoxin antibody). Thus, indirect conjugation of the label with the antibody can be achieved.

In another embodiment of the invention, the anti-phosphotyrosine antibody need not be labeled, and the presence thereof can be detected using a labeled anti-antiphosphotyrosine antibody (e.g. anti-mouse anti-phosphotyrosine antibody conjugated with HRPO).

In the preferred embodiment, the anti-phosphotyrosine antibody is labeled with an enzymatic label which catalyzes a color change of a substrate (such as tetramethyl benzimidine [TMB], or orthophenylene diamine [OPD]). Thus, the use of radioactive materials is avoided. A color change 5 of the reagent can be determined spectrophotometrically at a suitable wavelength (e.g. 450nm for TMB and 490nm for OPD, with a reference wavelength of 650 nm).

### 3. Intracellular Kinase Activity

The assay described herein is also useful for measuring 10 phosphorylation and/or activation of intracellular kinases (e.g. cytoplasmic tyrosine kinases and/or cytoplasmic serine-threonine kinases). Phosphorylation of these molecules can occur as a consequence of trans-phosphorylation of the intracellular kinase by a kinase receptor or "receptor complex" (which comprises one or more kinase receptors residing 15 in a cell membrane). Examples of intracellular tyrosine kinases include insulin receptor substrate I (IRS-1), Shc, Ras and GRB2, for example. Antibodies to human Shc, human Ras and GRB2 can be obtained commercially from UBI, NY, which can be used as capture agents for these tyrosine 20 kinases. Examples of intracellular serine-threonine kinases include MEK and MAPK.

In order to measure phosphorylation of these kinases, the procedure is essentially as described above except that a chimera of the intracellular kinase and the flag polypeptide is normally formed (i.e. a "kinase construct"). Alternatively, the cell has an endogenous 25 intracellular kinase or is transformed with nucleic acid encoding an intracellular kinase of interest. Generally, a eukaryotic cell will be transformed with nucleic acid encoding a kinase construct. Upon expression of the nucleic acid, the kinase or kinase construct will reside intracellularly (i.e. in the cytoplasm). The cells comprising the kinase 30 or kinase construct are subjected to the KIRA as discussed above. Exposure to the agonist may result in trans-phosphorylation of the intracellular kinase which can be quantified in the ELISA as elaborated above. The capture agent in the ELISA binds to either the intracellular kinase or to the flag polypeptide.

### 35 4. Serine-Threonine Kinase Activity

This assay is further useful for measuring phosphorylation and/or activation of serine-threonine kinases. The term "serine-threonine kinase" refers to a kinase which phosphorylates a substrate which has at least one

phosphate accepting alcohol group. The serine-threonine kinase is usually a "receptor" insofar as it has a ligand-binding ECD, TM domain and ICD. The ICD usually comprises a catalytic kinase domain and generally has one or more phosphate accepting serine and/or threonine residues. Examples of 5 intracellular serine-threonine kinases include MEK and MAPK. See section 3 above for a discussion as to measuring phosphorylation of intracellular serine-threonine kinases. Examples of serine-threonine kinase receptors include *daf-1*, activin type II receptor (ActR-II), activin type IIB receptor (ActR-IIB), TGF- $\beta$  type II receptor (T $\beta$ R-II), activin receptor-like 10 kinase (ALK) -1, -2, -3, -4 and TGF- $\beta$  type I receptor (T $\beta$ R-1)/ALK-5. See ten Dijke et al., *supra*. The serine-threonine kinase assay is essentially the same as described above for tyrosine kinases, except that phosphorylation is quantified using anti-phosphoserine and/or anti-phosphothreonine antibodies. Anti-phosphoserine and anti-phosphothreonine 15 monoclonal antibodies can be purchased from Sigma Immuno Chemicals, St Louis, MO, for example.

##### 5. Phosphatase Activity

Phosphatase activity can similarly be measured using the assay described herein. Phosphatase enzymes are able to dephosphorylate 20 phosphorylated tyrosine, serine and/or threonine residues (i.e. liberate inorganic phosphate from phosphoric esters of such amino acid residues). Generally the phosphatase enzyme is specific for either tyrosine residues or serine-threonine residues but sometimes can dephosphorylate tyrosine, serine and threonine residues. Sometimes "endogenous" phosphatase activity 25 is measured and this refers to the activity of phosphatase enzyme(s) which exist in nature in a selected cell.

In order to quantify endogenous phosphatase activity, cells possessing at least one phosphatase are stimulated in the presence and absence of one or more phosphatase inhibitors. Examples of protein tyrosine 30 phosphatase (PTPase) inhibitors include sodium orthovanadate and sodium molybdate (Sigma Chemical Co., St. Louis, MO). ICN Biochemicals supply okadaic acid which is a serine-threonine phosphatase inhibitor. As a general proposition, between about 1-10 $\mu$ M phosphatase inhibitor can be added to each well of the assay plate. In all other respects, the assay is 35 performed essentially as discussed above. Thus, the ability of endogenous phosphatases to dephosphorylate a kinase in the selected cell can be quantified.

In the preferred embodiment, a phosphatase enzyme of interest can be studied. Examples of protein tyrosine phosphatases (PTPases) include PTP1B, PTPMEG, PTP1c, Yop51, VH1, cdc25, CD45, HLAR, PTP18, HPTPa and DPTP10D. See Zhang and Dixon, Adv. Enzym., 68: 1-36 (1994). Examples of protein serine-threonine phosphatases include PP1, PP2A, PP2B and PP2C. See Meth. Enzym., ed Hunter & Sefton, Academic press, New York, 201:389-398 (1991). These proteins can be purchased commercially or made using the recombinant techniques described herein. To measure phosphatase activity, the KIRA ELISA can be performed essentially as described above with the following modifications. Following capture of the kinase or kinase construct (e.g. receptor construct) to the second solid phase and the washing step (to remove unbound cell lysate), the phosphatase of interest is added to the wells of the second assay plate and incubated with the adhering kinase or kinase construct. For example, between about 50-200  $\mu$ l of the phosphatase in a suitable dilution buffer (see Meth. Enzym., ed Hunter & Sefton, Academic press, New York, 201:416-440 [1991]) can be added to each well. This is generally followed by gentle agitation at room temperature (or 37°C) for between about 30 min to 2 hours to allow the phosphatase to dephosphorylate the kinase. Following washing to remove the phosphatase, the decreased degree of phosphorylation of the kinase relative to the control (i.e. no phosphatase added) is quantified by ELISA as described earlier herein.

#### 6. Kits

As a matter of convenience, the reagents can be provided in a kit, i.e., a packaged combination of reagents, for combination with the analyte in assaying the ability of the analyte to activate or prevent activation of a rPTK of interest. The components of the kit will be provided in predetermined ratios. Thus, a kit will comprise the specific second solid phase for the assay as well as the anti-flag polypeptide capture agent either packaged separately or captured to the second solid phase (e.g. a microtiter plate). Usually, other reagents, such as the anti-phosphotyrosine antibody labelled directly or indirectly with an enzymatic label will also be provided in the kit. Where the detectable label is an enzyme, the kit will include substrates and cofactors required by the enzyme (e.g. a substrate precursor which provides the detectable chromophore or fluorophore). In addition, other additives may be included such as stabilizers, buffers (e.g. a block buffer and a lysis buffer) and the like. Conveniently, the kit can also supply the homogeneous population

of cells which have been transformed with the receptor construct. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided 5 as dry powders, usually lyophilized, including excipients which on dissolution will provide a reagent solution having the appropriate concentration. The kit also suitably includes instructions for carrying out the KIRA ELISA.

#### 7. Uses for the Assay

10 This application provides two assays which are useful for reliable, sensitive and quantitative detection of kinase activation. The first assay can be used where a kinase receptor-specific capture antibody having the desired characteristics herein described is available or has been prepared. The second assay is a generic assay which enables activation of any kinase 15 receptor to be measured via the use of a flag polypeptide and a capture agent which binds specificity thereto.

These assays are useful for identifying novel agonists/antagonists for a selected kinase receptor. Also, the assay provides a means for studying ligand-receptor interactions (i.e., mechanism studies). Also the 20 presence of an endogenous receptor in a selected cell line can be quantified using the assay. The assays are further useful for identifying the presence of a ligand for a selected kinase receptor in a biological sample and, e.g., establishing whether a growth factor has been isolated following a purification procedure. It is desirable to have an assay for 25 measuring the ability of these growth factors to activate their respective receptors.

The assay also has clinical applications for detecting the presence of a ligand for a selected rPTK (e.g. the insulin receptor) in a biological sample taken from a human and thus patients having elevated or depressed 30 levels of the ligand can be identified. This is particularly desirable where elevated or depressed levels of the ligand cause a pathological condition. Accordingly, candidates for administration of the selected ligand (e.g. insulin) can be identified through this diagnostic method. It is possible, using the assay disclosed herein, to assay the pK of agonists 35 or antagonists administered to a patient. This assay also facilitates the detection of shed receptor in a biological sample.

The assay is also useful to quantify phosphatase activity of endogenous phosphatases or, in the preferred embodiment, a phosphatase of

interest. This can be used for screening phosphatase inhibitors, for example.

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes 5 only, and are not intended to limit the scope of the present invention in any way.

All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

10

EXAMPLE 1KIRA ELISA of the HER2 Receptor

The assay system described in this example was developed to measure the extent of autophosphorylation as a result of the interactions between the HER2 receptor and its specific activator, heregulin (HRG). The 15 overexpression of p185<sup>HER2</sup> has been correlated with poor clinical outcome in a number of epithelial-derived cancers. Heregulin and its rodent homologue, neu differentiation factor (NDF), were originally purified based on their ability to stimulate the autophosphorylation of a 185 kDa protein in the breast carcinoma cell lines MCF-7 and MDA-453, respectively. In this 20 embodiment of the invention, the cell line expressing the tyrosine kinase receptor DNA (either endogenous or transformed) is adherent and there is an antibody (e.g. monoclonal or affinity purified polyclonal) capable of specifically binding the receptor such that it neither stimulates autophosphorylation in the absence of ligand nor suffers impaired binding 25 due to the presence of bound ligand. Standard curve preparations and many samples may easily be run simultaneously in replicate and at several dilutions using this assay, readily allowing quantitation of ligand activity in a large number of unknown samples.

(i) Capture agent preparation

30 Polyclonal anti-HER2 antibody was isolated from pooled immune sera from New Zealand White rabbits immunized with the extracellular domain of the HER2 molecule (Fendly et al., Journal of Biological Response Modifiers 9:449-455 [1990]). The rHER2 ECD specific antibodies were affinity purified using an FPLC (Pharmacia Biotech, Inc, Piscataway, NJ) with an 35 affinity column generated from rHER2 ECD conjugated to Avidgel F (Bioprobe International, Inc, Tustin, CA). The resulting purified antibody stock was

0.829 mg/ml in phosphate buffered saline (PBS), pH 7.4, and was stored as 0.5 ml aliquots at -20°C.

(ii) Anti-phosphotyrosine antibody preparation

Monoclonal anti-phosphotyrosine, clone 4G10, was purchased from 5 Upstate Biologicals, Inc (UBI, Lake Placid, NY) and biotinylated using long-arm biotin-N-hydroxysuccinamide (Biotin-X-NHS, Research Organics, Cleveland, OH).

(iii) Ligand

The recombinant truncated form of  $\beta$ iheregulin (MW= 7.88 Kd) 10 corresponding to residues 177-244 (HRG $\beta$ 1<sub>177-244</sub>) was produced in *E. coli* and purified to homogeneity as described in Holmes et al., *Science*, 256: 1205-1210 (1992) and was stored at 4°C as an 89.7  $\mu$ M stock solution in 50 mM Tris/HCl, pH 7.5.

(iv) Adherent Cells

15 MCF-7 (ATCC-HTB 22), an adherent cell line isolated from a human breast adenocarcinoma, was obtained from American Type Culture Collection (ATCC, Rockville, MD). MCF-7 cells have been shown to produce measurable levels of surface p185<sup>HER2</sup> by both FACS and ELISA analysis. The cells were maintained in 150 cm<sup>2</sup> tissue culture flasks (Corning Inc, Corning, NY) and 20 utilized when at cell densities of 60 % to 75 % confluency. For the assay, 2 x 10<sup>5</sup> cells were seeded per well in flat-bottom microtiter plates (Falcon 3072, Becton Dickinson Labware, Lincoln Park, NJ) cultured overnight at 37°C in 5 % CO<sub>2</sub>. Cells were grown in F12/DMEM 50:50 Gibco as a custom formulation (Gibco/BRL, Life Technologies, Grand Island, NY). The medium 25 was supplemented with 10 % FBS (HyClone, Logan, Utah), 25 mM HEPES (Gibco) and 2 mM L-glutamine (Gibco).

(v) KIRA ELISA

MCF-7 cells (2 x 10<sup>5</sup>) in 100  $\mu$ l media were added to each well in a flat-bottom-96 well culture plate and cultured overnight at 37°C in 5% CO<sub>2</sub>. 30 The following morning the well supernatants were decanted, and the plates were lightly tamped on a paper towel. 50  $\mu$ l of media containing either experimental samples or the recombinant HRG $\beta$ 1<sub>177-244</sub> standards (3000, 1000, 333, 111, 37, 12, 4, and 0 pM) was then added to each well. The cells were stimulated at 37°C for 30 min., the well supernatants were decanted, and 35 the plates were once again lightly tamped on a paper towel. To lyse the cells and solubilize the receptors, 100  $\mu$ l of lysis buffer was added to each well. Lysis buffer consisted of 150 mM NaCl containing 50 mM HEPES (Gibco), 0.5 % Triton-X 100 (Gibco), 0.01 % thimerosal, 30 KIU/ml aprotinin

(ICN Biochemicals, Aurora, OH), 1mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; ICN Biochemicals), 50  $\mu$ M leupeptin (ICN Biochemicals), and 2 mM sodium orthovanadate ( $Na_3VO_4$ , Sigma Chemical Co, St. Louis, MO), pH 7.5. The plate was then agitated gently on a plate shaker 5 (Bellco Instruments, Vineland, NJ) for 60 min. at room temperature.

While the cells were being solubilized, an ELISA microtiter plate (Nunc Maxisorp, Inter Med, Denmark) coated overnight at 4°C with the affinity-purified polyclonal anti-HER2 ECD (1.0  $\mu$ g/ml in 50 mM carbonate buffer, pH 9.6, 100  $\mu$ l/well) was decanted, tamped on a paper towel and 10 blocked with 150  $\mu$ l/well of Block Buffer [PBS containing 0.5 % BSA (Intergen Company, Purchase, NY) and 0.01 % thimerosal] for 60 min. at room temperature with gentle agitation. After 60 minutes, the anti-HER2 ECD coated plate was washed 6 times with wash buffer (PBS containing 0.05 % Tween-20 and 0.01 % thimerosal) using an automated plate washer (ScanWasher 15 300, Skatron Instruments, Inc, Sterling, VA).

The lysate containing solubilized p185<sup>HER2</sup> from the cell-culture microtiter well was transferred (85  $\mu$ l/well) to anti-rHER2 ECD coated and blocked ELISA well and was incubated for 2 h at room temperature with gentle agitation. The unbound receptor was removed by washing with wash 20 buffer and 100  $\mu$ l of biotinylated 4G10 (anti-phosphotyrosine) diluted 1:2000 in dilution buffer (PBS containing 0.5 % BSA, 0.05 % Tween-20, 5 mM EDTA, and 0.01 % thimerosal), i.e. 400pg/ml, was added to each well. After incubation for 2 h at room temperature the plate was washed and 100  $\mu$ l of 25 HRPO-conjugated streptavidin (Zymed Laboratories, S. San Francisco, CA) diluted 1:10000 in dilution buffer was added to each well. The plate was incubated for 30 minutes at room temperature with gentle agitation. The free avidin-conjugate was washed away and 100  $\mu$ l freshly prepared substrate solution (tetramethyl benzidine [TMB]; 2-component substrate kit; Kirkegaard and Perry, Gaithersburg, MD) was added to each well. The 30 reaction was allowed to proceed for 10 minutes, after which the color development was stopped by the addition of 100  $\mu$ l/well 1.0 M  $H_3PO_4$ . The absorbance at 450 nm was read with a reference wavelength of 650 nm (ABS<sub>450/650</sub>), using a vmax plate reader (Molecular Devices, Palo Alto, CA) controlled with a Macintosh Centris 650 (Apple Computers, Cupertino, CA) 35 and DeltaSoft software (BioMetallics, Inc, Princeton, NJ).

The standard curve shown in Figure 7 was generated by stimulating MCF-7 cells with 3000, 1000, 333, 111, 37, 12, 4, or 0 pM HRG $\beta$ 1<sub>177-244</sub> and presented as pM HRG $\beta$ 1<sub>177-244</sub> vs. mean ABS<sub>450/650</sub>  $\pm$  sd using the DeltaSoft

program. Sample concentrations were obtained by interpolation of their absorbance on the standard curve and are expressed in terms of pM HRG $\beta$ 1<sub>177-244</sub> activity.

When the data were fitted to a 4-parameter nonlinear least squares equation, they resulted in a correlation coefficient of 0.9998. For the data shown in Figure 7, the EC<sub>50</sub> of receptor activation by HRG $\beta$ 1<sub>177-244</sub> was 373 pM. To demonstrate the highly reproducible nature of the p185<sup>HER2</sup> KIRA ELISA, seven standard curves were generated over the period of one month and the EC<sub>50</sub>'s are averaged. This gives an EC<sub>50ave</sub> for HRG $\beta$ 1<sub>177-244</sub> of 360 ± 10 40 pM (average ± SD).

(vi) Intra- and inter-assay precision and assay specificity

The intra-assay variability was determined by performing the p185<sup>HER2</sup> KIRA ELISA on three separate days. For each test, the standard curve is run in triplicate. Controls with HRG $\beta$ 1<sub>177-244</sub> corresponding to high (1000 pM), mid (200 pM) and low (40 pM) were assayed in 24 replicates. The ABS<sub>450/650</sub> of the individual test samples were converted to pM HRG $\beta$ 1<sub>177-244</sub> activity and the 24 converted values for each test concentration were averaged. The data are expressed as averaged value and % coefficient of variation (%cv; [(intra-assay standard deviation/intra-assay averaged calculated value) × 100]. See Table 1A below.

Table 1  
Intra- and Inter-assay Variation  
A. Intra-assay Precision (n=24 per test)

	High Value <sup>a</sup>		Mid Value		Low Value	
	Average Value (pM)	% cv <sup>b</sup>	Average Value (pM)	% cv	Average Value (pM)	% cv
25	Test #1	1256	19.5%	209	10.8%	33
	Test #2	1078	10.0%	196	5.1%	38
	Test #3	999	14.3%	196	6.3%	35

## B. Inter-assay Precision (n=3)

Average Value (pM)	%cv <sup>c</sup>	Average Value (pM)	% cv	Average Value (pM)	% cv
1100	4.3%	200	6.3%	34	9.0%

5      <sup>a</sup>Expected high value: 1000 pM; mid value: 200 pM; low value: 40 pM

b Intra-assay % cv determined as intra-assay sd/intra-assay average x 100

c Inter-assay % cv determined as inter-assay sd/inter-assay average x 100

10     The intra-assay variability of the KIRA ELISA was within acceptable limits despite the fact that the assay actually consists of both bioassay and ELISA components. The coefficients of variance (%) for the highest values were under 20% and for the mid and low values were at or under 10%.

15     The inter-assay variability was determined by averaging the values from upper-most three adjacent wells (of the 24 wells run) for a given sample concentration from each run. The three separate averages for each test concentration were then averaged. The data were expressed as averaged value and %cv [(inter-assay standard deviation/inter-assay averaged value) x 100]. See Table 1B. above. The inter-assay variability of the KIRA ELISA was within acceptable limits.

20     In order to confirm the specificity of the assay, MCF-7 cells were stimulated with either HRG $\beta$ 1<sub>177-244</sub> at 3000, 1000, 333, 111, 37, 12, 4 or 0 pM or insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), or insulin at 30000, 10000, 3333, 1111, 370, 120, 40 or 0 pM. The p185<sup>HER2</sup> KIRA ELISA was then performed as described above. The results are depicted in Figure 8.

25     The p185<sup>HER2</sup> KIRA ELISA was clearly specific for heregulin. While HRG $\beta$ 1<sub>177-244</sub> induced normal receptor stimulation and autophosphorylation, the closely related EGF gives only a slight stimulation ( $OD_{450/650} = 0.239$ ) at the highest concentration tested (100 nM). Since EGF-R is produced in MCF-7 cells, this signal is likely due to EGF receptor transphosphorylation of p185<sup>HER2</sup>. Neither insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF) nor insulin have any detectable effect on the MCF-7 p185<sup>HER2</sup> KIRA ELISA, the latter despite the fact that MCF-7 cells produce active insulin receptors.

30     The results presented in this example demonstrate that the KIRA ELISA is a useful method for assaying ligand activation of a kinase receptor, e.g., heregulin activation of the p185<sup>HER2</sup> receptor. Levels of receptor activation in terms of tyrosine phosphorylation are easily quantified and an EC<sub>50</sub> for a given ligand is readily determined. One potential use for this

assay would be to screen compounds for receptor agonist or antagonist activities. The potential throughput for this assay greatly surpasses that of Western blot analysis. Since the cell-culture portion of the assay is conducted in 96-well plates, many samples may be run in replicate at 5 different dilutions at one time in a one-day assay.

EXAMPLE 2

KIRA ELISA of the Rse Receptor

Mark et al., Journal of Biological Chemistry 269(14):10720-10728 (1994) describe isolation of the Rse receptor protein tyrosine kinase from 10 human and murine tissues. This Rse receptor with a carboxyl-terminal flag polypeptide (i.e. Rse.gD) was subjected to the KIRA ELISA described herein. The experimental procedure is outlined below.

(i) Capture agent preparation

Monoclonal anti-gD (clone 5B6) was produced against a peptide from 15 Herpes simplex virus glycoprotein D (Paborsky et al., Protein Engineering 3(6):547-553 [1990]). The purified stock preparation was adjusted to 3.0mg/ml in phosphate buffered saline (PBS), pH 7.4 and 1.0ml aliquots were stored at -20°C.

(ii) Anti-phosphotyrosine antibody preparation

20 Monoclonal anti-phosphotyrosine, clone 4G10, was purchased from Upstate Biologicals, Inc (UBI, Lake Placid, NY) and biotinylated using long-arm biotin-N-hydroxysuccinamide (Biotin-X-NHS, Research Organics, Cleveland, OH).

(iii) Ligand

25 Since the endogenous ligand for the Rse receptor was not available, an agonist antibody for the Rse receptor was prepared which forms the ligand for the KIRA ELISA described in this Example. To generate the agonist antibody, a Rse.IgG chimera was generated. Briefly, the coding sequence of the ECD of Rse was fused to that of the human IgG-γ<sub>1</sub> heavy chain in a multi-step process. PCR was used to generate a fragment with a unique BstEII site 3' to the coding sequences of the Rse amino acid 428. The PCR product was joined to the human IgG-γ<sub>1</sub> heavy chain cDNA through a unique BstEII site in that construct (Mark et al., J. Cell. Biol., 267: 26166-26171 [1992]). The resulting construct (termed pRK.bpTK3.IgG.fusion) 30 contained the coding sequences for amino acids 375-428 of Rse joined to those encoding human IgG-γ<sub>1</sub> heavy chain. The remaining portion of the Rse 35

ECD (amino acids 1-374) was then added by linkage through the Bam HI site in pRK.bpTK3.IgG.fusion to yield pRK.Rse.IgG.

To generate stable cell populations expressing Rse.IgG, the cDNA encoding Rse.IgG was subcloned into the episomal CMV-driven expression 5 plasmid pcIS.EBON, a pRK5 derivative disclosed in Cachianes et al., Bio. Techniques, 15: 225-259 (1993). Human fetal kidney 293 cells (obtained from ATCC, 12301 Parklawn Drive, Rockville, MD, USA) were transfected by the calcium phosphate technique. Cell monolayers were incubated for four hours in the presence of the DNA precipitate, glycerol shocked, and cultured in 10 F12:DMEM (1:1) containing 2mM glutamine, 10% fetal bovine serum, penicillin and streptomycin. After 48 hours, populations were replated in media containing G418 to select for a stable population of cells. Conditioned media was collected from cells expressing Rse.IgG nucleic acid that have been cultured in serum-free media for 72 hours in the absence of G418.

15 Rse.IgG was purified by affinity chromatography on a protein A column using procedures as described by Chamow, S.M., et al., Biochemistry, 29:9885-9891 (1990) with the following minor modifications. Conditioned media collected from cells expressing the Rse.IgG was adjusted to 0.1 M citrate pH 6.0 and loaded directly onto a protein A column (Repligen). The 20 column was washed with 0.1 M citrate, pH 6.0, and was eluted with 3 M MgCl<sub>2</sub> with 10% glycerol. Fractions were pooled and desalting on a PD-10 column, dialyzed and concentrated against PBS. Protein concentrations were determined by an ELISA against human IgG (Fc). The protein was analyzed for purity by Coomassie staining of PAGE gels.

25 Polyclonal antibodies were generated in New Zealand white rabbits against the Rse.IgG formed as described above. 4 $\mu$ g of Rse.IgG in 100 $\mu$ L PBS was emulsified with 100 $\mu$ L Freund's adjuvant (complete adjuvant for the primary injection and incomplete adjuvant for all boosts). For the primary immunization and the first boost, the protein was injected directly into 30 the popliteal lymph nodes (Sigel et al., Methods Enzymol., 93, 3-12 [1983]). For subsequent boosts, the protein was injected into subcutaneous and intramuscular sites. 1.3  $\mu$ g protein/kg body weight was injected every 3 weeks with bleeds taken 1 and 2 weeks following each boost. The polyclonal antisera generated was then precipitated in 50% ammonium 35 sulphate.

The resultant, purified polyclonal antisera is called "19B" herein. To confirm the ability of the 19B antisera to induce autophosphorylation of the Rse receptor, serum starved 3T3.gD.R11 cells (transformed with

nucleic acid encoding the Rse receptor with an amino terminal gD flag polypeptide [i.e. gD.Rse] using the techniques described in Mark et al., *Journal of Biological Chemistry* 269(14):10720-10728 [1994]) or NIH3T3 cells were exposed to pre-immune serum or 19B polyclonal antisera at a 1:200 dilution for 10 minutes. The gD.Rse protein was immunoprecipitated from extracts using the anti-gD monoclonal antibody 5B6. Proteins were fractionated on 7% SDS-PAGE under reducing conditions and transferred to nitrocellulose. Phosphorylation of Rse was detected with labelled anti-phosphotyrosine antibody. Treatment of the 3T3.gD.R11 cells with 19B antisera stimulated the phosphorylation of the 140kD gD.Rse protein. This increase was not observed in cells treated with pre-immune sera.

The purified 19B polyclonal antisera was stored at 4°C as an 2.8mg/ml stock solution in PBS, pH 7.5.

(iv) Preparation of Rse.gD nucleic acid

15 Synthetic double stranded oligonucleotides were used to reconstitute the coding sequence for the C-terminal 10 amino acids (880 - 890) of human Rse and add an additional 21 amino acids containing an epitope for the antibody 5B6 and a stop codon. The final sequence of the synthetic portion of the fusion gene was:

20 coding strand:

5' - TGCAGCAAGGGCTACTGCCACACTCGAGCTGCGCAGATGCTAGCCTCAAGATGGCT G ATCCAAATCGATTCCGGCAAAGATCTTCGGTCCTGTAGAAGCT-3' (SEQ ID NO: 10)

noncoding (anti-sense) strand:

5' - AGCTTCTACAGGACCGGAAGATCTTGCCGGAAATCGATTTGGATCAGCCATCTT G

25 AGGCTAGCATCTGCGCAGCTCGAGTGTGGCAGTAGCCCTTGCTGCA-3' (SEQ ID NO: 11).

The synthetic DNA was ligated with the cDNA encoding amino acids 1-880 of human Rse at the PstI site beginning at nucleotide 2644 of the published human Rse cDNA sequence (Mark et al., *Journal of Biological Chemistry* 269(14):10720-10728 [1994]) and HindIII sites in the polylinker 30 of the expression vector pSVI7.ID.LL (See Figure 16; SEQ ID NO: 9) to create the expression plasmid pSV.ID.Rse.gD. Briefly, the expression plasmid comprises a dicistronic primary transcript which contains sequence encoding DHFR bounded by 5' splice donor and 3' splice acceptor intron splice sites, followed by sequence that encodes the Rse.gD. The full 35 length (non-spliced) message contains DHFR as the first open reading frame and therefore generates DHFR protein to allow selection of stable transformants.

(v) Cell transformation

dp12. CHO cells (EP 307,247 published 15 March 1989) were electroporated with 20 µgs of pSV.ID.Rse.gD which had been linearized at a unique NotI site in the plasmid backbone. The DNA was ethanol precipitated after phenol/chloroform extraction and was resuspended in 20µl 1/10 Tris EDTA. Then, 10µg of DNA was incubated with 10<sup>7</sup> CHO.dp12 cells in 1 ml of PBS on ice for 10 min. before electroporation at 400 volts and 330µf. Cells were returned to ice for 10 min. before being plated into non-selective medium. After 24 hours cells were fed nucleoside-free medium to select for stable DHFR+ clones.

(vi) Selection of transformed cells for use in the KIRA ELISA

To identify a cell line that expresses Rse.gD nucleic acid, candidate clones were screened by fluorescence activated cell sorting (FACS) analysis using the polyclonal antiserum 19B generated as described above, which recognizes epitopes in the extracellular domain of Rse. See Figure 5, step (b).

To confirm that clones that scored positive in the FACS assay express full-length Rse.gD nucleic acid, cell lysates were prepared (Lokker et al., EMBO J, 11:2503-2510 [1992]) and solubilized Rse.gD was immunoprecipitated with the 19B antisera. The immunoprecipitated proteins were fractionated under reducing conditions using 7% PAGE, blotted onto nitrocellulose and then probed with the anti-gD 5B6 antibody which was detected with a horseradish peroxidase conjugated anti-mouse IgG antibody. See Figure 5, step (c). The ability of Rse.gD in cell clones to be activated to undergo autophosphorylation in response to the 19B agonistic antibody was determined. Briefly, serum starved dp. CHO cells transformed with Rse.gD nucleic acid as described above were exposed to pre-immune or 19B antisera at a 1:200 dilution for 10 min. The Rse.gD protein was immunoprecipitated from extracts using the anti-gD 5B6 monoclonal antibody. Proteins were fractionated on 7% SDS-PAGE under reducing conditions and transferred to nitrocellulose. Phosphorylation of Rse was detected with labelled antiphosphotyrosine antibody. See Figure 5, step (d).

(vii) Media

Cells were grown in F12/DMEM 50:50 (Gibco/BRL, Life Technologies, Grand Island, NY). The media was supplemented with 10% diafiltered FBS (HyClone, Logan, Utah), 25mM HEPES and 2mM L-glutamine.

(viii) KIRA ELISA

Rse.gD transformed dp12. CHO cells (EP 307,247 published 15 March 1989) were seeded ( $5 \times 10^4$  per well) in the wells of a flat-bottom-96 well culture plate in 100  $\mu$ l media and cultured overnight at 37°C in 5% CO<sub>2</sub>. The 5 following morning the well supernatants were decanted, and the plates were lightly tamped on a paper towel. 100  $\mu$ l of media containing either experimental samples or 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200 or 0 diluted, anti-Rse agonist polyclonal antibody (19B pAb) was then added to each well. The cells were stimulated at 37°C for 30 min., the well 10 supernatants were decanted, and the plates were once again lightly tamped on a paper towel. To lyse the cells and solubilize the receptors, 100  $\mu$ l of lysis buffer was added to each well. Lysis buffer consisted of 150 mM NaCl containing 50 mM HEPES (Gibco), 0.5 % Triton-X 100 (Gibco), 0.01 % thimerosal, 30 KIU/ml aprotinin (ICN Biochemicals, Aurora, OH), 1mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; ICN Biochemicals), 50  $\mu$ M leupeptin (ICN Biochemicals), and 2 mM sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>; Sigma Chemical Co, St. Louis, MO), pH 7.5. The plate 15 was then agitated gently on a plate shaker (Bellco Instruments, Vineland, NJ) for 60 min. at room temperature.

20 While the cells were being solubilized, an ELISA microtiter plate (Nunc Maxisorp, Inter Med, Denmark) coated overnight at 4°C with the 5B6 monoclonal anti-gD antibody (0.5  $\mu$ g/ml in 50 mM carbonate buffer, pH 9.6, 100  $\mu$ l/well) was decanted, tamped on a paper towel and blocked with 150  $\mu$ l/well of Block Buffer [PBS containing 0.5 % BSA (Intergen Company, 25 Purchase, NY) and 0.01 % thimerosal] for 60 min. at room temperature with gentle agitation. After 60 minutes, the anti-gD 5B6 coated plate was washed 6 times with wash buffer (PBS containing 0.05 % Tween-20 and 0.01 % thimerosal) using an automated plate washer (ScanWasher 300, Skatron Instruments, Inc, Sterling, VA).

30 The lysate containing solubilized Rse.gD from the cell-culture microtiter well was transferred (85  $\mu$ l/well) to anti-gD 5B6 coated and blocked ELISA well and was incubated for 2 h at room temperature with gentle agitation. The unbound Rse.gD was removed by washing with wash buffer and 100  $\mu$ l of biotinylated 4G10 (anti-phosphotyrosine) diluted 35 1:2000 in dilution buffer (PBS containing 0.5 % BSA, 0.05 % Tween-20, 5 mM EDTA, and 0.01 % thimerosal), i.e. 400 pg/ml was added to each well. After incubation for 2 h at room temperature the plate was washed and 100  $\mu$ l of HRPO-conjugated streptavidin (Zymed Laboratories, S. San Francisco, CA)

diluted 1:10000 in dilution buffer was added to each well. The plate was incubated for 30 minutes at room temperature with gentle agitation. The free avidin-conjugate was washed away and 100  $\mu$ l freshly prepared substrate solution (tetramethyl benzidine [TMB]; 2-component substrate kit; 5 Kirkegaard and Perry, Gaithersburg, MD) was added to each well. The reaction was allowed to proceed for 10 minutes, after which the color development was stopped by the addition of 100  $\mu$ l/well 1.0 M H<sub>3</sub>PO<sub>4</sub>. The absorbance at 450 nm was read with a reference wavelength of 650 nm (ABS<sub>450/650</sub>), using a vmax plate reader (Molecular Devices, Palo Alto, CA) 10 controlled with a Macintosh Centris 650 (Apple Computers, Cupertino, CA) and DeltaSoft software (BioMetallics, Inc, Princeton, NJ).

The standard curve shown in Figure 10 was generated by stimulating Rse.gD transformed CHO cells with 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200 or 0 diluted, anti-Rse agonist antibody (19B) and presented as 15 1/dilution anti-Rse agonist antibody (19B) vs. mean ABS<sub>450/650</sub>  $\pm$  sd using the DeltaSoft program.

The results presented in this example demonstrate that the KIRA ELISA is a useful method for assaying ligand activation of a receptor construct having a carboxyl terminal flag polypeptide, e.g., activation of Rse.gD. 20 Levels of receptor activation in terms of tyrosine phosphorylation are easily quantified and an EC<sub>50</sub> for a given ligand (e.g. an agonist antibody for the receptor) is readily determined.

#### EXAMPLE 3

##### KIRA ELISA of the trk A, B and C Receptors

25 Neurotrophins belong to a family of small, basic proteins which play a crucial role in the development and maintenance of the nervous system. The first identified and probably best understood member of this family is nerve growth factor (NGF). See U.S. Patent No. 5,169,762, issued December 8, 1992. Recently, sequentially related but distinct polypeptides with 30 similar functions to NGF have been identified. For example, brain-derived neurotrophic factor (BDNF), now also referred to as neurotrophin-2 (NT2), was cloned and sequenced by Leibrock et al. (Nature, 341: 149-152 [1989]). Several groups identified a neurotrophic factor originally called neuronal factor (NF), and now referred to as neurotrophin-3 (NT3). (Ernfors et al., 35 Proc. Natl. Acad. Sci. USA, 87: 5454-5458 [1990]; Höhn et al., Nature, 344: 339 [1990]; Maisonpierre et al., Science, 247: 1446 [1990]; Rosenthal et al., Neuron, 4: 767 [1990]; Jones and Reichardt, Proc. Natl. Acad. Sci.

USA, 87: 8060-8064 [1990]; Kaisho et al., FEBS Lett., 266: 187 [1990]). Neurotrophins-4 and -5 (NT4 and NT5) have been recently added to the family (Hallbook et al., Neuron, 6: 845-858 [1991]; Berkmeier et al., Neuron, 7: 857-866 [1991]; Ip et al., Proc. Natl. Acad. Sci. USA, 89: 3060-3064 5 [1992]).

Neurotrophins, similarly to other polypeptide growth factors, affect their target cells through interactions with cell surface rPTKs (called Trk receptors). The first member of the trk receptor family, trkA, was initially identified as the result of an oncogenic transformation caused 10 by the translocation of tropomyosin sequences onto its catalytic domain. Later work identified trkA as a signal transducing receptor for NGF. Subsequently, two other related receptors, mouse and rat trkB (Klein et al., EMBO J., 8: 3701-3709 [1989]; Middlemas et al., Mol. Cell. Biol., 11: 143-153 [1991]; EP 455,460 published 6 November 1991) and porcine, mouse 15 and rat trkC (Lamballe et al., Cell, 66: 967-979 [1991]; EP 522,530 published 13 January 1993), were identified as members of the trk receptor family. The structures of the trk receptors are quite similar, but alternate splicing increases the complexity of the family by giving rise 20 to two known forms of trkA, three known forms of trkB (two without functional tyrosine kinase domains) and at least four forms of trkC (several without functional tyrosine kinase domain, and two with small inserts in the tyrosine kinase domain). Human trk A, B and C receptor sequences are disclosed in U.S. Patent application Serial No. 08/215,139, filed March 18, 1994, specifically incorporated herein by reference.

25 The following KIRA ELISA was performed using trk A, B and C receptor constructs having amino-terminal flag polypeptides.

(i) Capture agent preparation

Monoclonal anti-gD (clone 5B6) was produced against a peptide from Herpes simplex virus glycoprotein D as discussed above in Example 2. The 30 purified stock preparation was adjusted to 3.0mg/ml in phosphate buffered saline (PBS), pH 7.4 and 1.0ml aliquots were stored at -20°C.

(ii) Anti-phosphotyrosine antibody preparation

Monoclonal anti-phosphotyrosine, clone 4G10, was purchased from Upstate Biologicals, Inc (UBI, Lake Placid, NY) and biotinylated using 35 long-arm biotin-N-hydroxysuccinamide (Biotin-X-NHS, Research Organics, Cleveland, OH).

(iii) Ligands

Nerve growth factor (NGF), neurotrophin 3 (NT3), and neurotrophin 5 (NT5) were prepared by recombinant techniques using the sequence data provided for each of these proteins in the above-mentioned references. The purified NGF, NT3 and NT5 were stored at 4°C as stock solutions (180 $\mu$ M, 5 8.8 $\mu$ M and 26.9 $\mu$ M, respectively) in PBS, pH 7.5.

(iv) Preparation of gD.trk nucleic acid

In order to express the various trk receptors with gD flags (i.e. gD.trk constructs), DNA constructs were made which encoded the signal and epitope of gD (see Paborsky et al., *supra*) fused to the amino terminus of 10 the various trk receptors. These were made by inserting the trk receptor and gD sequences into pRK5 or pRK7 (Suva et al., *Science*, 237: 893-896 [1987]) using standard molecular biology techniques, to generate the constructs shown in Figures 12-14. In addition to the gD.trk constructs, constructs were also made to express gD tagged trk.IgG fusion proteins 15 (i.e., gD.trk.IgG). DNA constructs encoding the chimeras of trk extracellular domain and IgG-1 Fc domains were made with the Fc region clones of human IgG-1 (Ashkenazi et al., *Immunoadhesins Intern. Rev. Immunol.*, 10: 219-227 [1993]). More specifically, the source of the IgG-1 encoding sequence was the CD4-IgG-1 expression plasmid pRKCD4<sub>2</sub>Fc<sub>1</sub> (Capon et 20 al., *Nature*, 334: 525 [1989]; Byrn et al., *Nature*, 344: 667 [1990]) containing a cDNA sequence encoding a hybrid polypeptide consisting of residues 1-180 of the mature human CD4 protein fused to human IgG-1 sequences beginning at aspartic acid 216 (taking amino acid 114 as the first residue of the heavy chain constant region; Kabat et al., *Sequences 25 of Proteins of Immunological Interest* 4th ed. [1987]), which is the first residue of the IgG-1 hinge after the cysteine residue involved in heavy-light chain bonding, and ending with residues 441 to include the CH2 and CH3 Fc domains of IgG-1. The CD4-encoding sequence was deleted from the expression plasmid pRKCD4<sub>2</sub>Fc<sub>1</sub> and the vector was fused to DNA encoding the 30 trk receptors, with the splice between aspartate 216 of the IgG-1 and valine 402 of trkA, threonine 422 of trkB, or threonine 413 of trkC. The gD tag was added to the amino terminus of each trk.IgG in the same way as for the gD.trk constructs.

(v) Cell transformation

35 Human embryonic kidney 293 cells (obtained from ATCC, Rockville, MD) were transiently transfected with the nucleic acid encoding gD.trk.IgG using a calcium phosphate protocol (Gorman, *DNA Cloning: A Practical Approach* [Glover, D., ed.] Vol II: 143-190, IRL Press, Washington DC).

After twelve hours, the transformed cells were rinsed three times with serum free F12/DMEM 50:50 media (Gibco) and then serum free media was added for a 48 hour collection.

Cell lines stably expressing each of the gD.trk constructs were made by co-transfected dp12. CHO cells (EP 307,247 published 15 March 1989) with the pRK plasmids encoding the gD tagged trk receptors and a plasmid encoding DHFR, again using calcium phosphate mediated transfection.

The media mentioned above (having the gD.trk.IgG) was used without further purification in binding assays to assess the effects of the presence of the gD flag polypeptide on neurotrophin binding to the gD.trk.IgG polypeptides. DNA encoding untagged trk.IgG polypeptide was run in parallel as a control. trk.IgG and gD tagged trk.IgG containing cell supernatants were prepared as described and used in competitive displacement assays with the appropriate iodinated neurotrophin. NGF is used as ligand for trkA, NT5 is used as ligand for trkB, and NT3 is used as a ligand for trkC. A summary of the results obtained is shown in the following table.

TABLE 1  
Binding of Neurotrophins to trk.IgG

	IC50 without gD	IC50 with gD
20      trkA	68.4+/-11.9 pM	68.8+/-3.0 pM
trkB	31.1+/-15.6 pM	12.1+/-18 pM
trkC	31.1+/-1.1 pM	30.2+/-0.7 pM

(vi) Selection of transformed cells for use in the KIRA ELISA

It was apparent from the preceding experiment that there was no observable change in the affinity of interaction of neurotrophins with their receptor due to the presence of the gD flag polypeptide on the amino terminus. Based on this result, cells were transformed with the gD.trk constructs for use in the KIRA ELISA using the techniques described in the previous section.

30      After two days, dp12. CHO cells (EP 307,247 published 15 March 1989) transformed with gD.trk constructs were selected for by growth in media without GHT, and after two weeks, growing cells were sorted by FACS analysis using the 5B6 monoclonal to select cells expressing the gD flag polypeptide on their surface. gD positive cells were cloned by plating at 35 limiting dilution and resultant colonies were then rescreened by FACS

analysis (using the anti-gD 5B6 monoclonal antibody), neurotrophin binding (as discussed above), tyrosine phosphorylation indicated by Western blot using an anti-phosphotyrosine antibody, gD expression by Western blot using the anti-gD 5B6 antibody, and immunocytochemistry using the 5B6 antibody. 5 Clones which were positive were then recloned by limiting dilution and were subjected to the KIRA ELISA as described below.

(vii) Media

Cells were grown in F12/DMEM 50:50 (Gibco/BRL, Life Technologies, Grand Island, NY). The media was supplemented with 10% diafiltered FBS 10 (HyClone, Logan, Utah), 25mM HEPES and 2mM L-glutamine.

(viii) KIRA ELISA

gD.trk transformed dp12.CHO cells (EP 307,247 published 15 March 1989) were seeded ( $5 \times 10^4$  per well) in a flat-bottom-96 well culture plate in 100  $\mu$ l media and cultured overnight at 37°C in 5% CO<sub>2</sub>. The following 15 morning the well supernatants were decanted, and the plates were lightly tamped on a paper towel. 100 $\mu$ l of media containing either experimental samples or the recombinant purified NGF, NT3, or NT5 standards (3000, 1000, 333, 111, 37, 12, 4, and 0 pM) was then added to each well. The cells were stimulated at 37°C for 30 min., the well supernatants were decanted, and 20 the plates were once again lightly tamped on a paper towel. To lyse the cells and solubilize the receptors, 100  $\mu$ l of lysis buffer was added to each well. Lysis buffer consisted of 150 mM NaCl containing 50 mM HEPES (Gibco), 0.5 % Triton-X 100 (Gibco), 0.01 % thimerosal, 30 KIU/ml aprotinin (ICN Biochemicals, Aurora, OH), 1mM 4-(2-aminoethyl)-benzenesulfonyl 25 fluoride hydrochloride (AEBSF; ICN Biochemicals), 50  $\mu$ M leupeptin (ICN Biochemicals), and 2 mM sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>; Sigma Chemical Co, St. Louis, MO). pH 7.5. The plate was then agitated gently on a plate shaker (Bellco Instruments, Vineland, NJ) for 60 min. at room temperature.

While the cells were being solubilized, an ELISA microtiter plate 30 (Nunc Maxisorp, Inter Med, Denmark) coated overnight at 4°C with the 5B6 monoclonal anti-gD antibody (0.5  $\mu$ g/ml in 50 mM carbonate buffer, pH 9.6, 100  $\mu$ l/well) was decanted, tamped on a paper towel and blocked with 150  $\mu$ l/well of Block Buffer [PBS containing 0.5 % BSA (Intergen Company, Purchase, NY) and 0.01 % thimerosal] for 60 min. at room temperature with 35 gentle agitation. After 60 minutes, the anti-gD 5B6 coated plate was washed 6 times with wash buffer (PBS containing 0.05 % Tween-20 and 0.01 % thimerosal) using an automated plate washer (ScanWasher 300, Skatron Instruments, Inc, Sterling, VA).

The lysate containing solubilized gD.trk from the cell-culture microtiter well was transferred (85  $\mu$ l/well) to anti-gD 5B6 coated and blocked ELISA well and was incubated for 2 h at room temperature with gentle agitation. The unbound gD.trk was removed by washing with wash buffer and 100  $\mu$ l of biotinylated 4G10 (anti-phosphotyrosine) diluted 1:2000 in dilution buffer (PBS containing 0.5 % BSA, 0.05 % Tween-20, 5 mM EDTA, and 0.01 % thimerosal), i.e., 400pg/ml, was added to each well. After incubation for 2 h at room temperature the plate was washed and 100  $\mu$ l of HRPO-conjugated streptavidin (Zymed Laboratories, S. San Francisco, CA) diluted 1:10000 in dilution buffer was added to each well. The plate was incubated for 30 minutes at room temperature with gentle agitation. The free avidin-conjugate was washed away and 100  $\mu$ l freshly prepared substrate solution (tetramethyl benzidine; 2-component substrate kit; Kirkegaard and Perry, Gaithersburg, MD) was added to each well. The reaction was allowed to proceed for 10 minutes, after which the color development was stopped by the addition of 100  $\mu$ l/well 1.0 M H<sub>3</sub>PO<sub>4</sub>. The absorbance at 450 nm was read with a reference wavelength of 650 nm (ABS<sub>450/650</sub>), using a vmax plate reader (Molecular Devices, Palo Alto, CA) controlled with a Macintosh Centris 650 (Apple Computers, Cupertino, CA) and DeltaSoft software (BioMetallics, Inc, Princeton, NJ).

The standard curves shown in Figures 15A-15C were generated by stimulating gD.trk transformed CHO cells with 3000, 1000, 333, 111, 37, 12, 4, and 0 pM NGF, NT3 or NT5 and were presented as pM neurotrophin vs. mean ABS<sub>450/650</sub>  $\pm$  sd using the DeltaSoft program. Sample concentrations were obtained by interpolation of their absorbance on the standard curve and are expressed in terms of pM neurotrophin activity.

The results presented in this example demonstrate that the KIRA ELISA is a useful method for assaying ligand activation of a receptor construct having an amino terminal flag polypeptide, e.g., activation of gD.trk receptor constructs. Levels of receptor activation in terms of tyrosine phosphorylation are easily quantified and an EC<sub>50</sub> for a given ligand is readily determined.

EXAMPLE 4

KIRA ELISA of the MPL/Rse Chimeric Receptor

The human MPL receptor has been disclosed by Vigon et al., PNAS, USA 89:5640-5644 (1992). A chimeric receptor comprising the ECD of the MPL receptor and the TM and ICD of Rse (Mark et al., *supra*) with a carboxyl-

terminal flag polypeptide (i.e. Rse.gD; see Example 2) was subjected to the KIRA ELISA described herein. The experimental procedure is outlined below. See also Figs. 16 and 17.

(i) Capture agent preparation

5 Monoclonal anti-gD (clone 5B6) was produced against a peptide from Herpes simplex virus glycoprotein D (Paborsky et al., Protein Engineering 3(6):547-553 [1990]). The purified stock preparation was adjusted to 3.0mg/ml in phosphate buffered saline (PBS), pH 7.4 and 1.0ml aliquots were stored at -20°C.

10 (ii) Anti-phosphotyrosine antibody preparation

Monoclonal anti-phosphotyrosine, clone 4G10, was purchased from UBI (Lake Placid, NY) and biotinylated using long-arm biotin-N-hydroxysuccinamide (Biotin-X-NHS, Research Organics, Cleveland, OH).

(iii) Ligand

15 The *MPL* ligand [de Sauvage et al., Nature 369: 533-538 (1994)] was prepared by recombinant techniques. The purified *MPL* ligand was stored at 4°C as a stock solution.

(iv) Preparation of *MPL/Rse.gD* nucleic acid

20 The expression plasmid pSV.ID.Rse.gD produced as described in Example 2 above was modified to produce plasmid pSV.ID.M.tmRd6 which contained the coding sequences of the ECD of human *MPL* (amino acids 1-491) fused to the transmembrane domain and intracellular domain of Rse.gD (amino acids 429-911). Synthetic oligonucleotides were used to join the coding sequence of a portion of the extracellular domain of human *MPL* to a portion of the Rse 25 coding sequence in a two step PCR cloning reaction as described by Mark et al. in J. Biol. Chem. 267: 26166-26171 (1992). Primers used for the first PCR reaction were M1 (5'-TCTCGCTACCGTTACAG - SEQ ID NO:12) and M2 (5'-CAGGTACCCACCAGGCGGTCTGGT - SEQ ID NO: 13) with a *MPL* cDNA template and R1 (5'-GGGCCATGACACTGTCAA - SEQ ID NO: 14) and R2 (5'- 30 GACCGCCACCGAGACCGCCTGGTGGGTACCTGTGGTCCTT - SEQ ID NO: 15) with a Rse cDNA template. The *Pvu*II-SmaI portion of this fusion junction was used for the construction of the full-length chimeric receptor.

(v) Cell transformation

dp12.CHO cells (EP 307,247 published 15 March 1989) were 35 electroporated with pSV.ID.M.tmRd6 which had been linearized at a unique NotI site in the plasmid backbone. The DNA was ethanol precipitated after phenol/chloroform extraction and was resuspended in 20μl 1/10 Tris EDTA. Then, 10μg of DNA was incubated with 10<sup>7</sup> CHO.dp12 cells in 1 ml of PBS on

ice for 10 min. before electroporation at 400 volts and 330 $\mu$ f. Cells were returned to ice for 10 min. before being plated into non-selective medium. After 24 hours cells were fed nucleoside-free medium to select for stable DHFR+ clones.

5           (vi) Selection of transformed cells for use in the KIRA ELISA

Clones expressing MPL/Rse.gD were identified by western-blotting of whole cell lysates post-fractionation by SDS-PAGE using the antibody 5B6 which detects the gD epitope tag.

(vii) Media

10          Cells were grown in F12/DMEM 50:50 (Gibco/BRL, Life Technologies, Grand Island, NY). The media was supplemented with 10% diafiltered FBS (HyClone, Logan, Utah), 25mM HEPES and 2mM L-glutamine.

(viii) KIRA ELISA

MPL/Rse.gD transformed dp12.CHO cells were seeded ( $3 \times 10^4$  per well) in 15 the wells of a flat-bottom-96 well culture plate in 100  $\mu$ l media and cultured overnight at 37°C in 5% CO<sub>2</sub>. The following morning the well supernatants were decanted, and the plates were lightly tamped on a paper towel. 50 $\mu$ l of media containing either experimental samples or 200, 50, 12.5, 3.12, 0.78, 0.19, 0.048 or 0 ng/ml MPL ligand was then added to each 20 well. The cells were stimulated at 37°C for 30 min., the well supernatants were decanted, and the plates were once again lightly tamped on a paper towel. To lyse the cells and solubilize the chimeric receptors, 100  $\mu$ l of lysis buffer was added to each well. Lysis buffer consisted of 150 mM NaCl 25 containing 50 mM HEPES (Gibco), 0.5 % Triton-X 100 (Gibco), 0.01 % thimerosal, 30 KIU/ml aprotinin (ICN Biochemicals, Aurora, OH), 1mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; ICN Biochemicals), 50  $\mu$ M leupeptin (ICN Biochemicals), and 2 mM sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>; Sigma Chemical Co, St. Louis, MO), pH 7.5. The plate was then agitated gently on a plate shaker (Bellco Instruments, Vineland, 30 NJ) for 60 min. at room temperature.

While the cells were being solubilized, an ELISA microtiter plate (Nunc Maxisorp, Inter Med, Denmark) coated overnight at 4°C with the 5B6 monoclonal anti-gD antibody (5.0  $\mu$ g/ml in 50 mM carbonate buffer, pH 9.6, 100  $\mu$ l/well) was decanted, tamped on a paper towel and blocked with 150 35  $\mu$ l/well of Block Buffer [PBS containing 0.5 % BSA (Intergen Company, Purchase, NY) and 0.01 % thimerosal] for 60 min. at room temperature with gentle agitation. After 60 minutes, the anti-gD 5B6 coated plate was washed 6 times with wash buffer (PBS containing 0.05 % Tween-20 and 0.01 %

thimerosal) using an automated plate washer (ScanWasher 300, Skatron Instruments, Inc, Sterling, VA).

The lysate containing solubilized *MPL/Rse.gD* from the cell-culture microtiter well was transferred (85  $\mu$ l/well) to anti-gD 5B6 coated and 5 blocked ELISA well and was incubated for 2 h at room temperature with gentle agitation. The unbound *MPL/Rse.gD* was removed by washing with wash buffer and 100  $\mu$ l of biotinylated 4G10 (anti-phosphotyrosine) diluted 1:18000 in dilution buffer (PBS containing 0.5 % BSA, 0.05 % Tween-20, 5 mM EDTA, and 0.01 % thimerosal), i.e. 56 ng/ml was added to each well. 10 After incubation for 2 h at room temperature the plate was washed and 100  $\mu$ l of HRPO-conjugated streptavidin (Zymed Laboratories, S. San Francisco, CA) diluted 1:60000 in dilution buffer was added to each well. The plate was incubated for 30 minutes at room temperature with gentle agitation. The free avidin-conjugate was washed away and 100  $\mu$ l freshly prepared 15 substrate solution (tetramethyl benzidine [TMB]; 2-component substrate kit; Kirkegaard and Perry, Gaithersburg, MD) was added to each well. The reaction was allowed to proceed for 10 minutes, after which the color development was stopped by the addition of 100  $\mu$ l/well 1.0 M H<sub>3</sub>PO<sub>4</sub>. The absorbance at 450 nm was read with a reference wavelength of 650 nm 20 (ABS<sub>450/650</sub>), using a vmax plate reader (Molecular Devices, Palo Alto, CA) controlled with a Macintosh Centris 650 (Apple Computers, Cupertino, CA) and DeltaSoft software (BioMetallics, Inc, Princeton, NJ).

The results demonstrated that *MPL* ligand was able to activate the *MPL/Rse.gD* chimeric receptor in a concentration-dependent and ligand-25 specific manner.

CLAIMS

1. A method for measuring autophosphorylation of a tyrosine kinase receptor comprising the steps of:
  - (a) coating a first solid phase with a homogeneous population of eukaryotic cells so that the cells adhere to the first solid phase, wherein, positioned in their membranes, the cells have a receptor construct comprising a flag polypeptide and the tyrosine kinase receptor;
  - (b) exposing the adhering cells to an analyte;
  - 10 (c) solubilizing the adhering cells, thereby releasing cell lysate therefrom;
  - (d) coating a second solid phase with a capture agent which binds specifically to the flag polypeptide so that the capture agent adheres to the second solid phase;
  - 15 (e) exposing the adhering capture agent to the cell lysate obtained in step (c) so that the receptor construct adheres to the second solid phase;
  - (f) washing the second solid phase so as to remove unbound cell lysate;
  - 20 (g) exposing the adhering receptor construct to an anti-phosphotyrosine antibody which identifies phosphorylated tyrosine residues in the tyrosine kinase receptor; and
  - (h) measuring binding of the anti-phosphotyrosine antibody to the adhering receptor construct.
- 25 2. The method of claim 1 wherein the cells are transformed with nucleic acid encoding the receptor construct prior to step (a).
3. The method of claim 1 wherein the cells comprise a mammalian cell line.
4. The method of claim 1 wherein the cells are adherent.
- 30 5. The method of claim 1 wherein the capture agent comprises a capture antibody.

6. The method of claim 1 wherein the first solid phase comprises a well of a first assay plate.
  7. The method of claim 6 wherein the first assay plate is a microtiter plate.
- 5 8. The method of claim 6 wherein between about  $1 \times 10^4$  to  $3 \times 10^5$  cells are added to the well in step (a).
9. The method of claim 1 wherein the second solid phase comprises a well of a second assay plate.
  10. The method of claim 1 wherein the cell lysate is not concentrated or clarified prior to step (e).
11. The method of claim 6 wherein step (c) comprises adding a lysis buffer to the well of the first assay plate and gently agitating the first assay plate.
12. The method of claim 11 wherein the lysis buffer comprises a solubilizing detergent.
13. The method of claim 1 wherein the anti-phosphotyrosine antibody is labelled.
14. The method of claim 13 wherein the label comprises an enzyme which is exposed to a color reagent and the color change of the color reagent is determined in step (h).
- 20 15. The method of claim 1 wherein the flag polypeptide is fused to the amino terminus of the tyrosine kinase receptor.
16. The method of claim 15 wherein the tyrosine kinase receptor is a trk A receptor, trk B receptor or trk C receptor.
- 25 17. The method of claim 1 wherein the flag polypeptide is fused to the carboxyl terminus of the tyrosine kinase receptor.

18. The method of claim 17 wherein the tyrosine kinase receptor is the Rse receptor.
19. The method of claim 17 wherein the receptor construct comprises the extracellular domain of a receptor of interest and the intracellular domain of the Rse receptor.  
5
20. The method of claim 19 wherein the receptor of interest is the MPL receptor.
21. The method of claim 20 wherein the receptor construct further comprises the transmembrane domain of the Rse receptor and the flag polypeptide comprises the gD polypeptide.  
10
22. The method of claim 1 wherein the analyte comprises an agonist for the tyrosine kinase receptor.
23. The method of claim 1 wherein the analyte comprises an antagonist for the tyrosine kinase receptor.
- 15 24. The method of claim 23 wherein the antagonist competitively inhibits binding or activation of the tyrosine kinase receptor by an agonist thereto and step (b) is followed by a step wherein the adhering cells are exposed to the agonist.
- 20 25. The method of claim 1 wherein the analyte is a composition which comprises an antagonist and an agonist for the receptor and the assay measures the ability of the antagonist to bind to the agonist and thereby reduce activation of the tyrosine kinase receptor by the agonist.
26. The method of claim 1 wherein a block buffer is added to the second solid phase following step (d).  
25
27. A method for measuring autophosphorylation of a tyrosine kinase receptor comprising the steps of:
  - (a) coating a well of a first assay plate with a homogeneous population of adherent cells so that the cells adhere to the

well, wherein the cells have a tyrosine kinase receptor positioned in the cell membranes thereof;

- (b) exposing the adhering cells to an analyte;
- (c) solubilizing the adhering cells thereby releasing cell lysate therefrom;
- 5 (d) coating a well of a second assay plate with a capture agent which binds specifically to the tyrosine kinase receptor so that the capture agent adheres to the well;
- (e) exposing the cell lysate obtained in step (c) to the adhering capture agent so that the tyrosine kinase receptor adheres to the well;
- 10 (f) washing the well so as to remove unbound cell lysate;
- (g) exposing the adhering tyrosine kinase receptor to an anti-phosphotyrosine antibody which binds selectively to phosphorylated tyrosine residues in the tyrosine kinase receptor;
- 15 (h) measuring binding of the anti-phosphotyrosine antibody to the adhering tyrosine kinase receptor.

28. The method of claim 27 wherein the tyrosine kinase receptor comprises  
20 the HER2 receptor.

29. A polypeptide comprising a flag polypeptide fused to the carboxyl terminus of the intracellular domain of the Rse receptor.

30. The polypeptide of claim 29 further comprising the transmembrane domain of the Rse receptor.

25 31. The polypeptide of claim 30 further comprising the extracellular domain of a receptor protein tyrosine kinase, other than the Rse receptor.

32. The polypeptide of claim 29 wherein the flag polypeptide comprises the gD flag.

30 33. A kit comprising a solid phase coated with a capture agent which binds specifically to a flag polypeptide.

34. The kit of claim 33 wherein the solid phase comprises a well of a microtiter plate.
  35. The kit of claim 33 further comprising a labeled anti-phosphotyrosine antibody.
- 5 36. The kit of claim 35 wherein the label comprises an enzyme.
37. The kit of claim 33 further comprising a cell transformed with a nucleic acid encoding a receptor construct.
  38. An assay for measuring phosphorylation of a kinase comprising the steps of:
    - 10 (a) coating a first solid phase with a homogeneous population of eukaryotic cells so that the cells adhere to the first solid phase, wherein the cells comprise a kinase construct comprising a flag polypeptide and the kinase;
    - (b) exposing the adhering cells to an analyte;
    - 15 (c) solubilizing the adhering cells, thereby releasing cell lysate therefrom;
    - (d) coating a second solid phase with a capture agent which binds specifically to the flag polypeptide so that the capture agent adheres to the second solid phase;
    - 20 (e) exposing the adhering capture agent to the cell lysate obtained in step (c) so that the kinase construct adheres to the second solid phase;
    - (f) washing the second solid phase so as to remove unbound cell lysate;
    - 25 (g) exposing the adhering kinase construct to an antibody which identifies phosphorylated residues in the kinase construct; and
    - (h) measuring binding of the antibody to the adhering kinase construct.
  39. The assay of claim 38 wherein the kinase is a receptor.

30 40. The assay of claim 38 wherein the kinase is a serine-threonine kinase.

41. The assay of claim 38 which measures phosphatase activity.
42. The assay of claim 41 wherein the cells further comprise a phosphatase and the assay further comprises the step of exposing the eukaryotic cells to a phosphatase inhibitor prior to step (c).
- 5 43. The assay of claim 41 which further comprises the steps in between steps (f) and (g) of exposing the adhering kinase construct to a phosphatase and then washing the second solid phase so as to remove unbound phosphatase.

1/70

FIG. I A

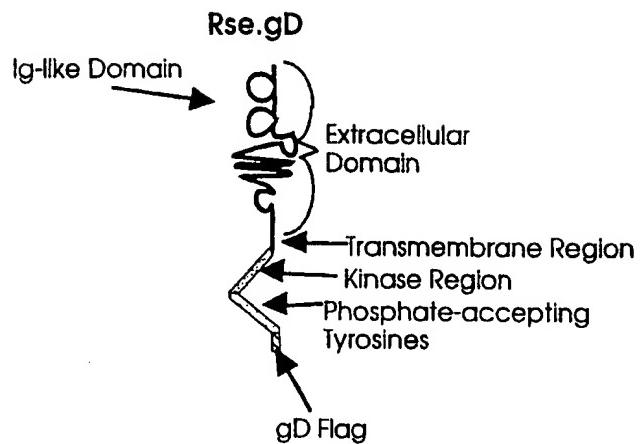


FIG. I B

Receptor ECD/Rse.gD Chimera

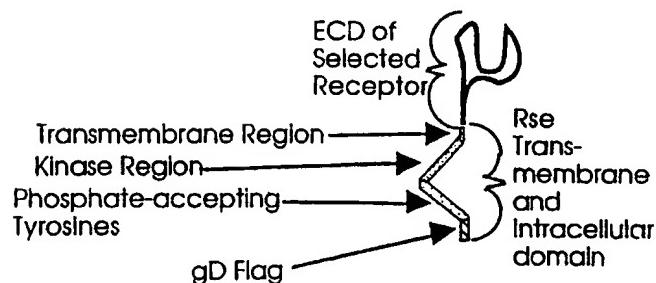
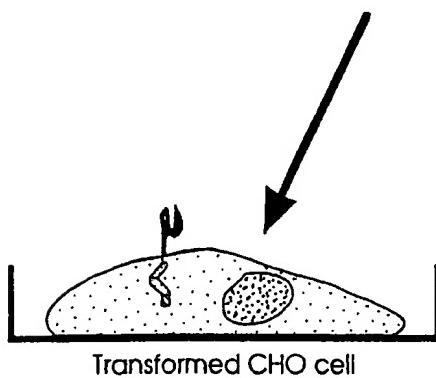


FIG. I C



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FIG. 2A

368 T Q D E L T V E G T R A N L T G W D P Q K D L I V R V C V S N A V  
 1101 AACCCAGGAT GAGCTGACAG TGGAGGGAC CAGGGCCAAT TTGACAGGCT AAAGGACCTG ATCGTACGTG TGTGCCTCTC CAATGCAGTT  
 transmembrane domain  
 401 G C G P W S Q P L V V S S H D R A G Q Q G P P H S R T S W V P V V L  
 1201 GGCTGGAC CCTGGAGTC GCCACTGGT GCCTCTCTC ATGACCGTGC AGCCAGCAG GGGCAGCAC ATCCCTGGTA CCTGTGGTCC  
 intracellular domain  
 435 G V L T A L V T A A A L I L R K R K E T R F G Q A F D S V  
 1301 TTGGTGCT AACGGCCCTG GTGACGGCTG CTGCCCCCTGGC CCTCATCCTG CTCGAAGA GACGGAAAGA GACGGGGTTT GGGCAAGCCT TTGACAGTGT

468 M A R G E P A V H F R A A R S F N R E R P E R I E A T L D S L G I  
 1401 CATGGCCGG GGAGGCCAG CGGTTCACTT CCGGGCAGCC CGGGAGGCC ATCGAGAAAG GCGCCAGGC ATCGAGGCCA CATGGACAG CTTGGGCCATC

501 S D E L K E K L E D V L I P E Q Q F T L G R M L G K G E F G S V R E  
 1501 AGCGATGAAAC TAAGGGAAA ACTGGAGGAT GTGCTCATCC CAGAGCAGA GTTCACCCCTG GGCCGGATGT TGGCAAAGG AGAGTTGGT TCAGTGGGG

535 A Q L K Q E D G S F V K V A V K M L K A D I I A S S D I E E F L R  
 1601 AGGCCAGGT GAAGCAAGAG GATGGCTCCT TTGTAAAGT GGCTGTAAAG ATGCTGAAG CTGACATCAT TGCCCTCAAGC GACATTGAAG AGTTCTCAG

568 E A A C M K E F D H P H V A K L V G V S L R S R A K G R L P I P M  
 1701 GGAAGCAGGT TGCATGAAG AGTTGACCA TCCACACGTG GCCAAACTTG TTGGGTAAG CCTCCGGAGC AGGGCTAAAG GCGCTCTCCC CATCCCCATG

601 V I L P F M K H G D L H A F L L A S R I G E N P F N L P L Q T L I R  
 1801 GTCATCTGC CCTCATGAA GCATGGGAC CTGCACTGCCT TCCATGCCT CCTGGGATT GGGGAGAAC CCTTAACCT ACCCTCTCAG ACCCTGATCC

635 F M V D I A C G M E Y L S S R N F I H R D L A A R N C M L A E D M  
 1901 GTTCAATGGT GGACATTGCC TCCGGCATGG AGTACCTGAG CTCTCGAAC TTCATCCACC GAGACCTGGC TGCTGGAAT TGCATGCTGG CAGAGGACAT

668 T V C V A D F G L S R K I Y S G D Y Y R Q G C A S K L P V K W L A  
 2001 GACACTGCTGACT TCGGACTCTC CCGGAAGATC TACAGTGGG ACTACTATCG TCAAGGCTGT GCCTCAAAAC TGCCTGCAA GTGGCTGCC

FIG.2B

3/70

701 L E S L A D N L Y T V Q S D V W A F G V T M W E I M T R G Q T P Y A  
 2101 CTGGAGGCC TGGCCGACAA CCTGTATACT GTGCAGAGTG ACGGTGTGGTC ACCATGTGGC AGATCATGAC ACGCCCAAGC  
 735 G I E N A E I Y N Y L I G G N R L K Q P P E C M E D V Y D L M Y Q  
 2201 CTGGCATCGA AACGCTGAG ATTTACAAC TACCTCATGG CGGGAACCGC CTGAAACAGC CTICCGGAGTG TATGGAGAC GTGTATGATC TCATGTACCA  
 768 C W S A D P K Q R P S F T C L R M E L E N I L G Q L S V L S A S Q  
 2301 GTGCTGGAGT GCTGACCCCCA AGCAGCCCCA GAGCTTACT TGCTCTCGAA TGGAACTGGA GAACATCTG GGCCAGCTGT CTGTGCTATC TGCCAGCCCC  
 801 D P L Y I N I E R A E E P T A G G S L E L P G R D Q P Y S G A G D G  
 2401 GACCCCTTAT ACATCAACAT CGAGAGAGCT GAGGACCCA CTGCGGGAGG CAGCCTGGAG CTACCTGGCA GGGATCAGCC CTACAGTGGG GCTGGGGATC  
 835 S G M G A V G G T P S D C R Y I L T P G G L A E Q P G Q A E H Q P  
 2501 GCAGTGGCAT GGCGGAGTG GCTGGCACTC CCAGTGACTG TCGGTACATA CTCACCCCCG GAGGGCTGGC TGAGCAGCCA GGGCAGGGAG AGCACCAAGC  
 868 E S P L N E T Q R L L L Q Q G L L P H S S C A D A S L K M A D P  
 2601 AGAGAGTCCC CTCATGAGA CACAGGGCT TTGCTGCTG CAGCAAGGGC TACTGCCACA CTCGAGCTGC GCAGATGCTA GCCTCAAGAT GGCTGATTCGA  
 901 N R F R G K D L P V L O  
 2701 AATGGATTCC GCGGAAAGA TCTTCCGGTC CTGTAGAAGC TT

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FIG.2C 4/70

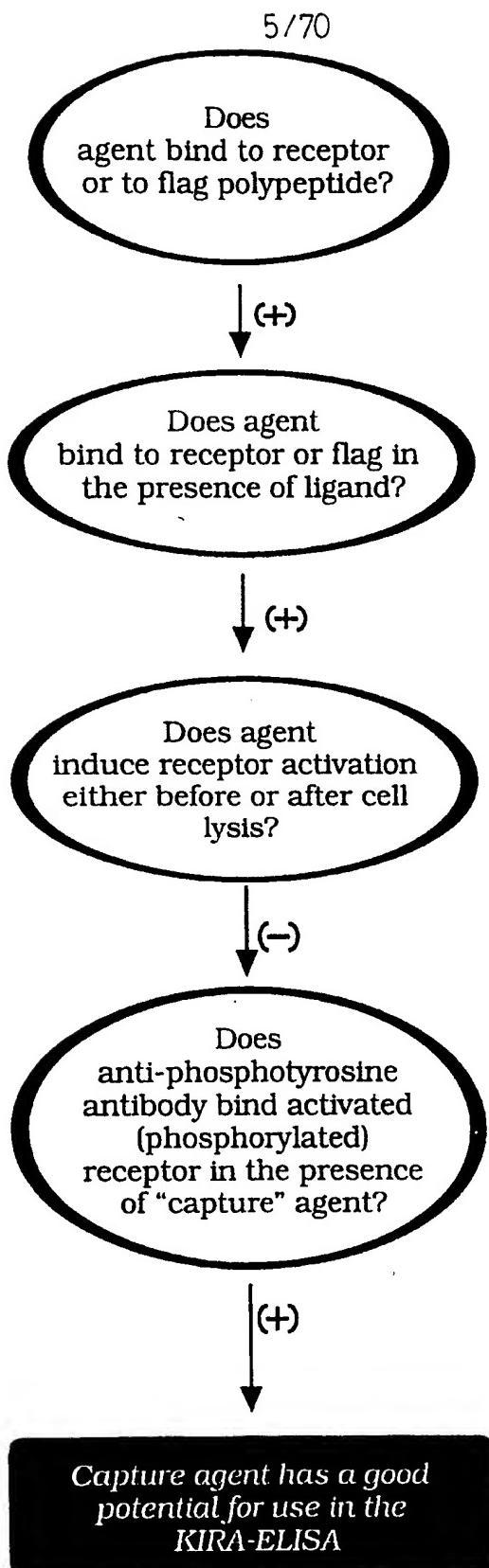


FIG.3

6/70

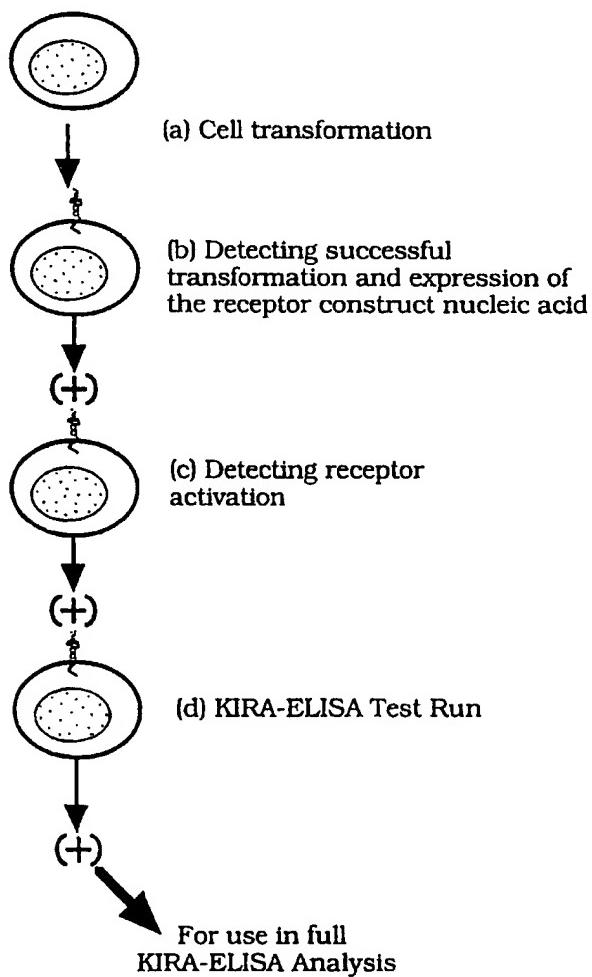
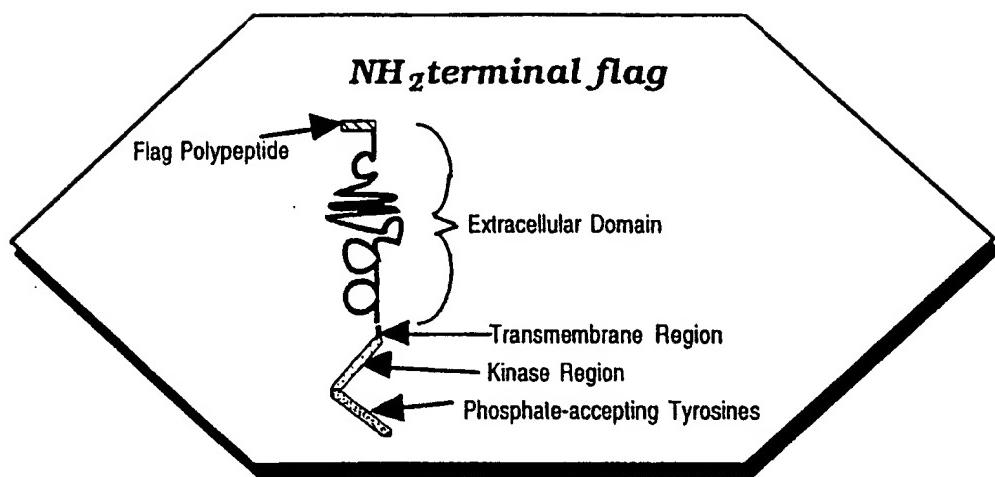
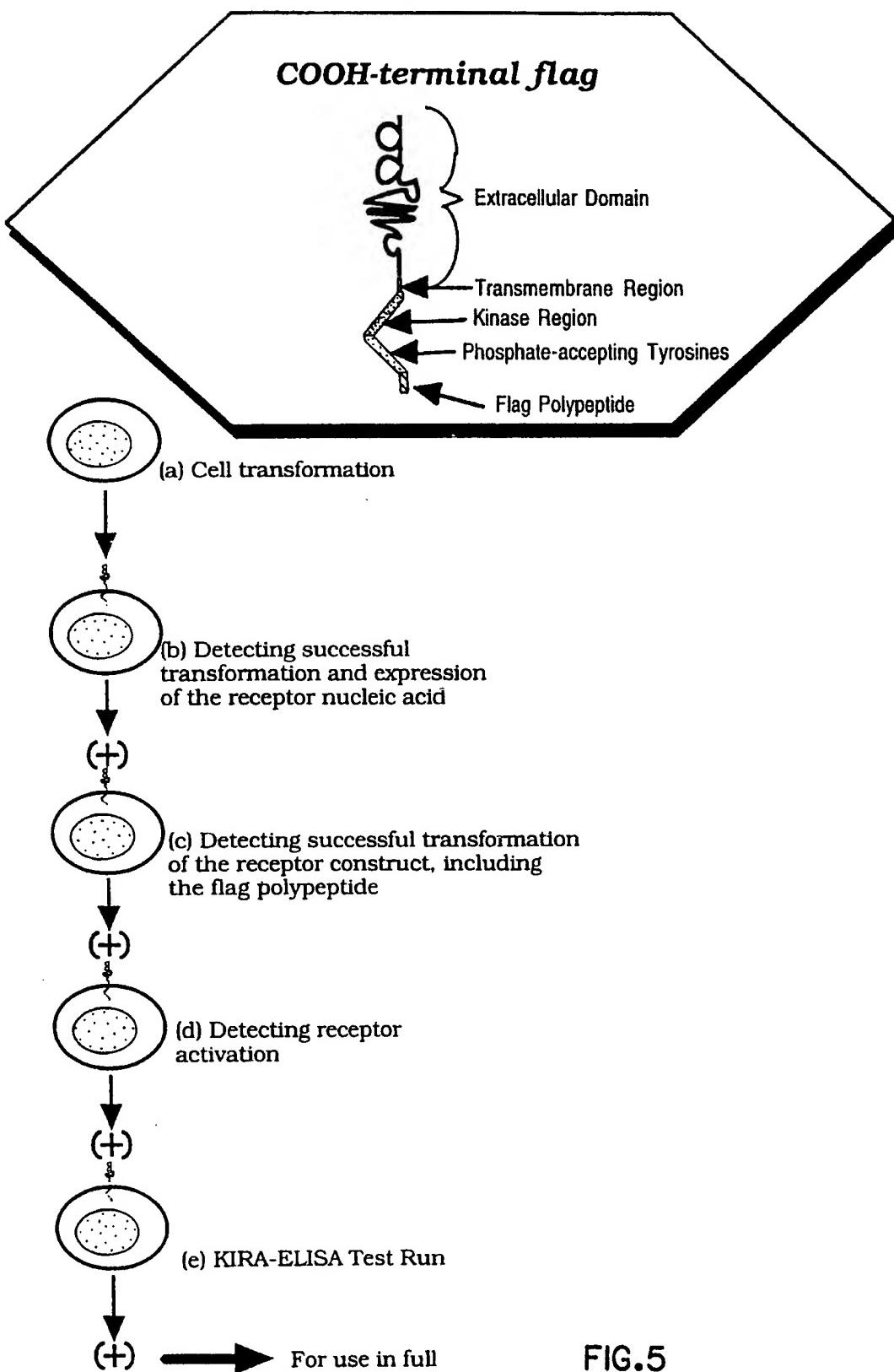


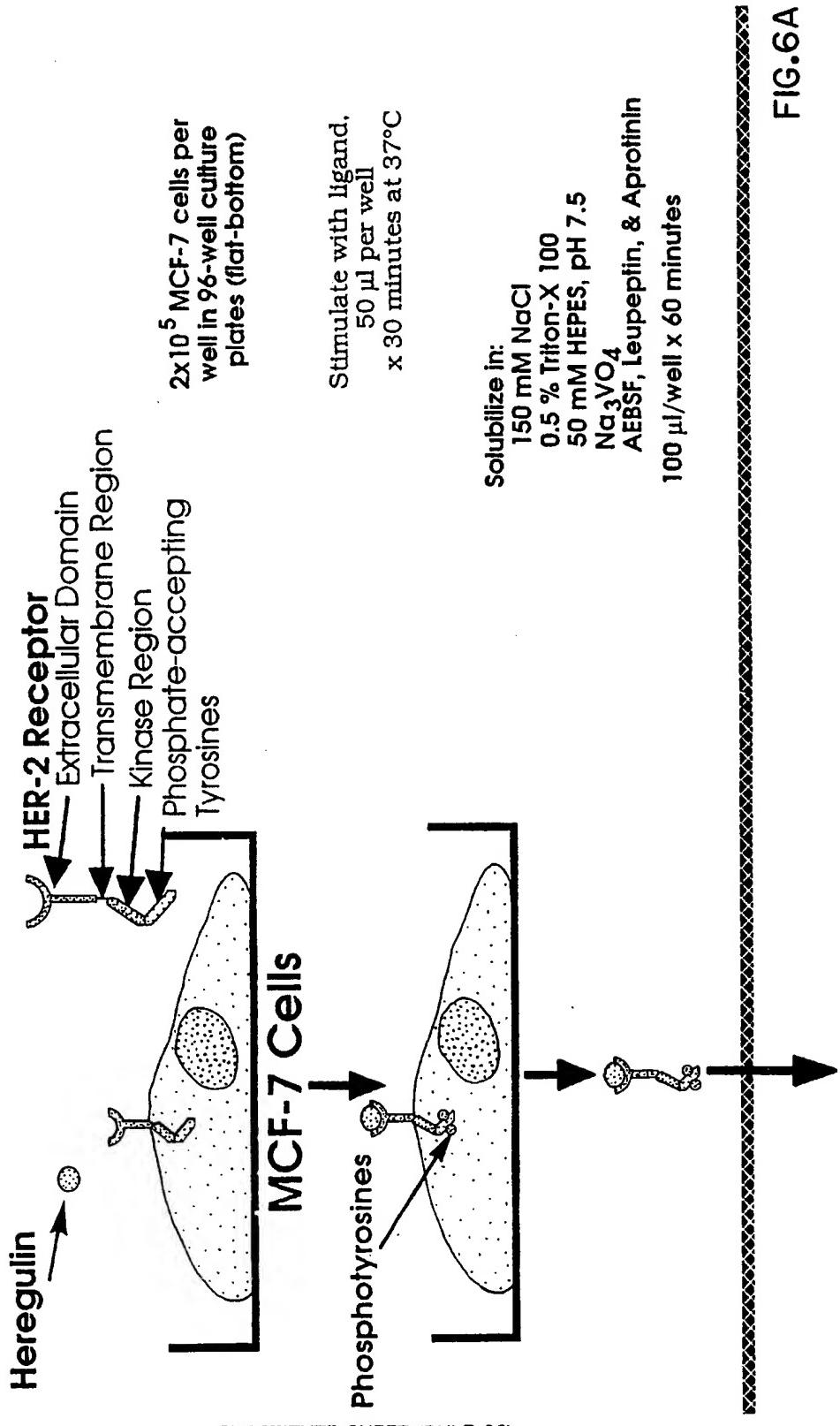
FIG.4  
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7/70



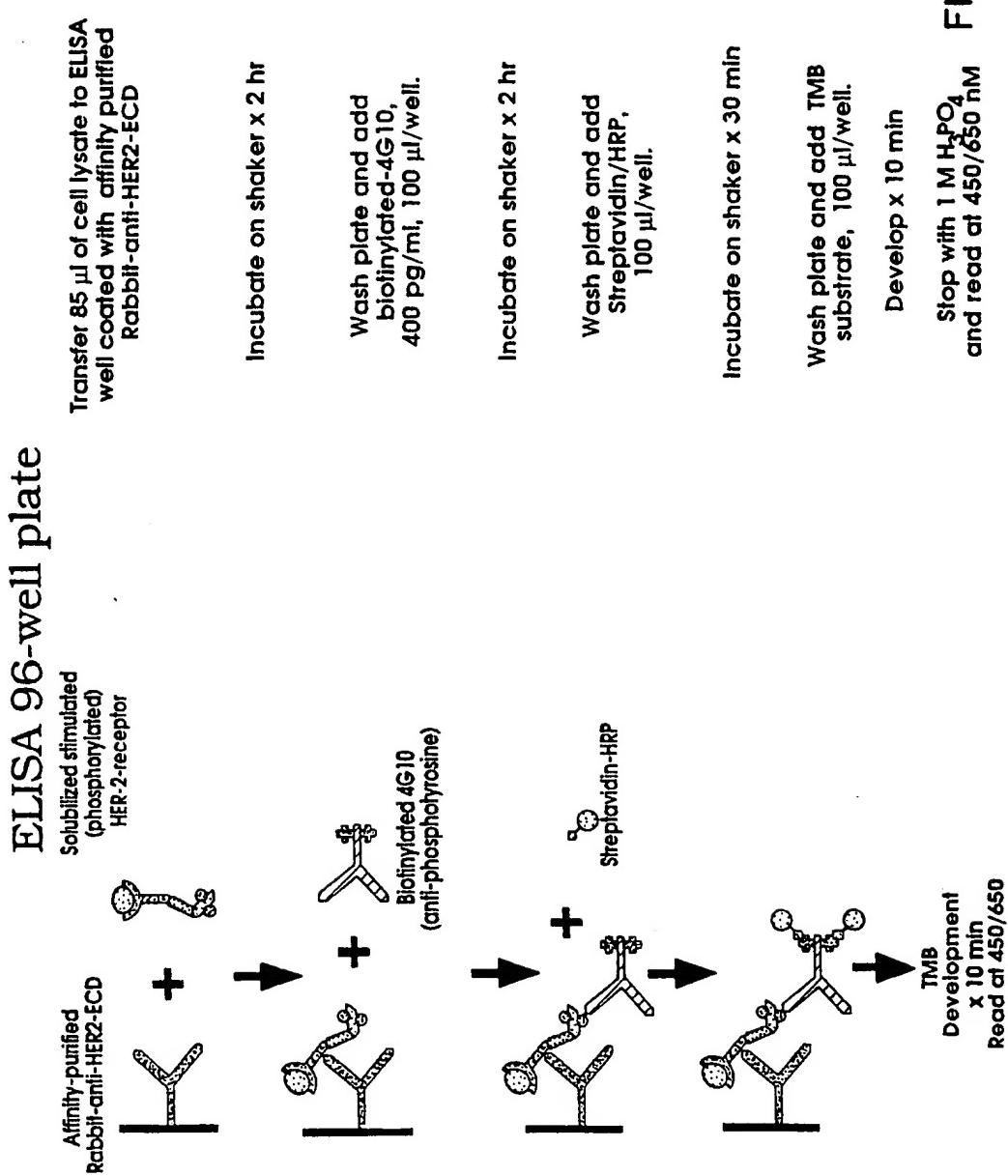
**FIG.5**  
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8/70



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9/70



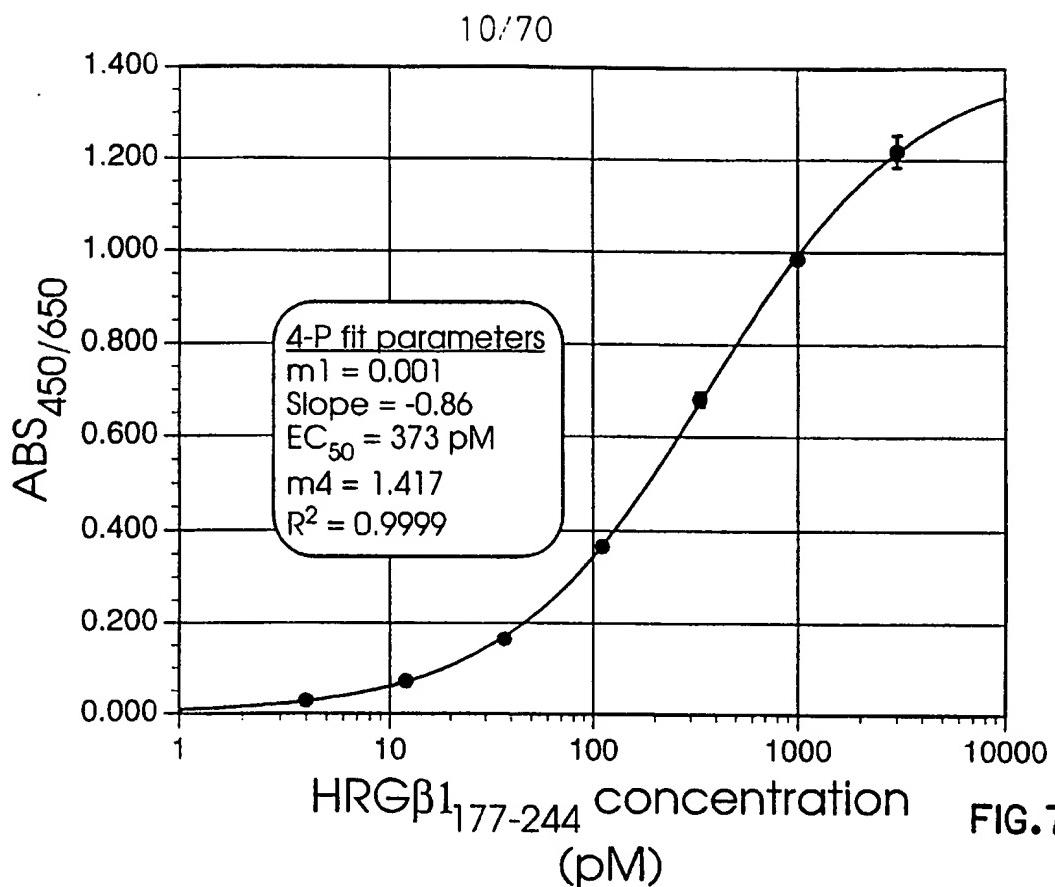


FIG.7

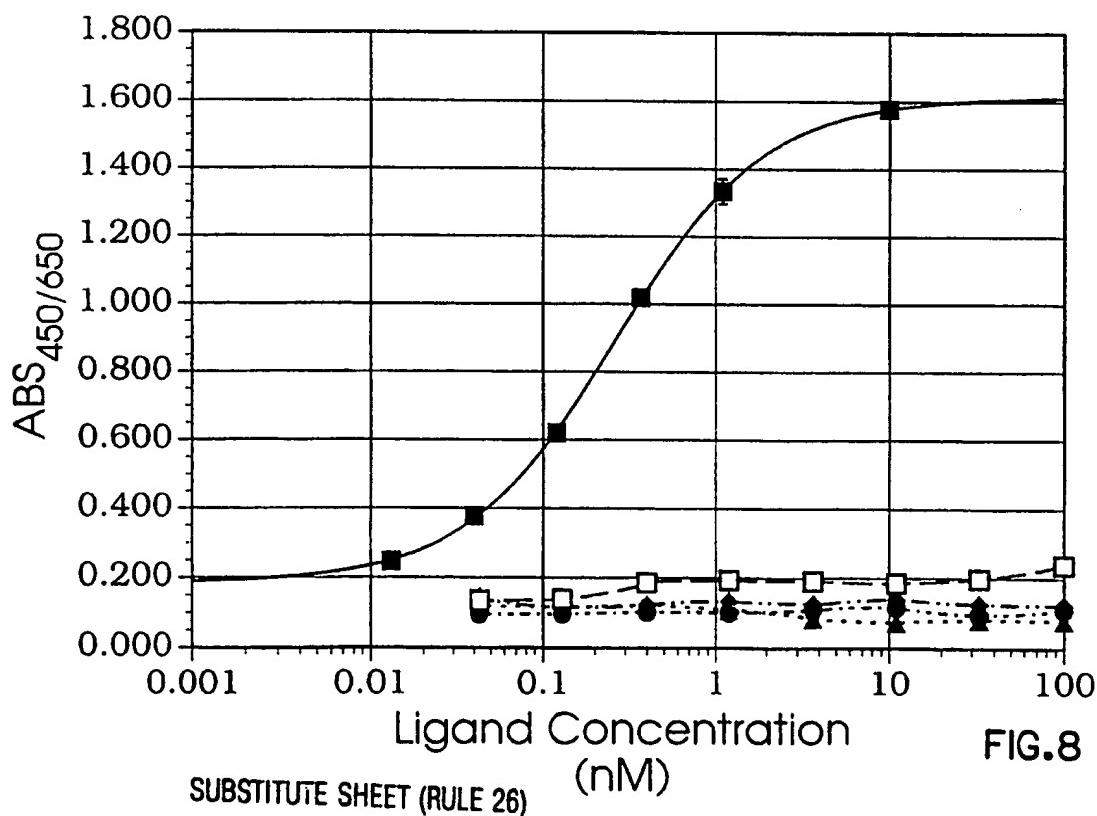
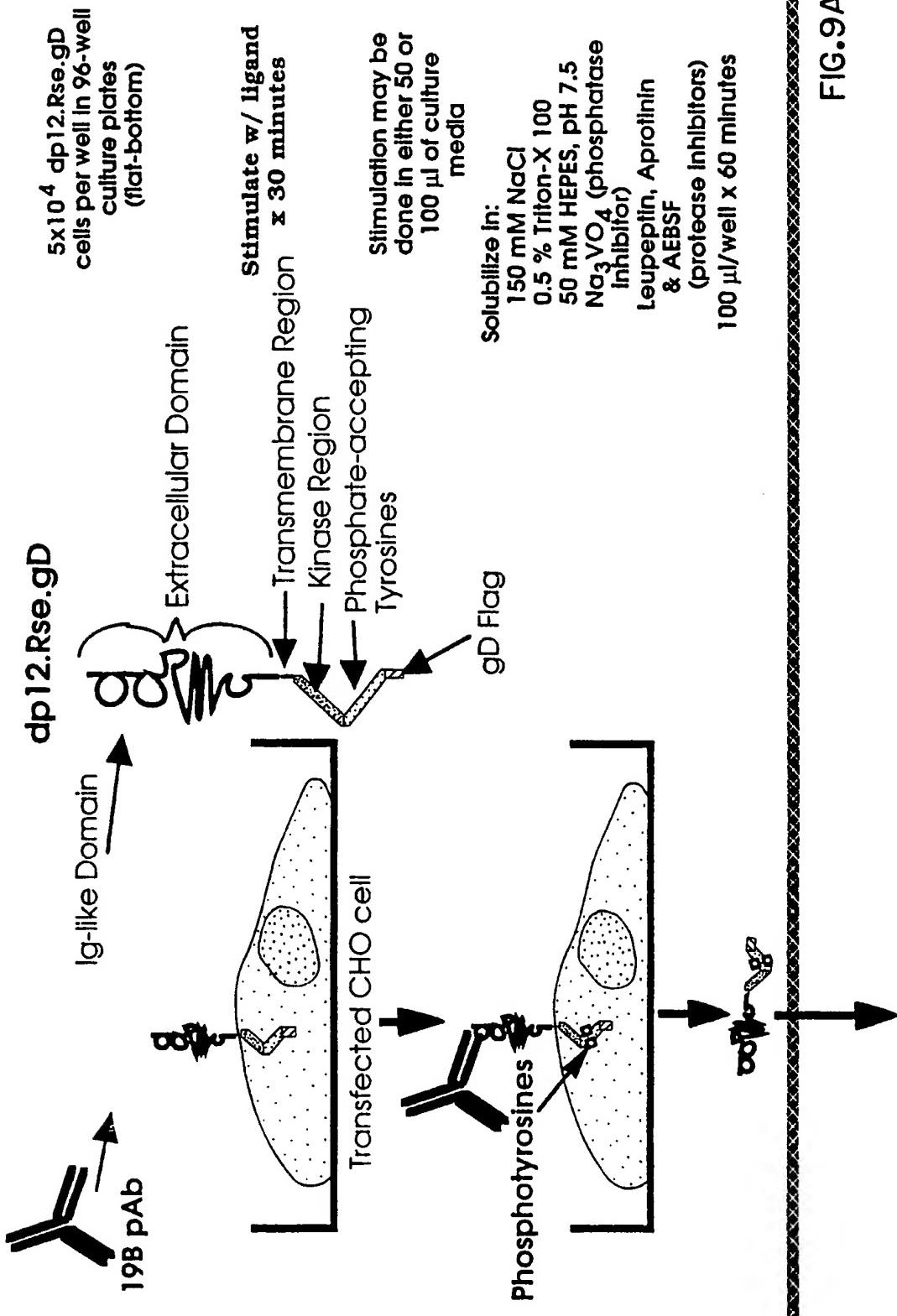


FIG.8

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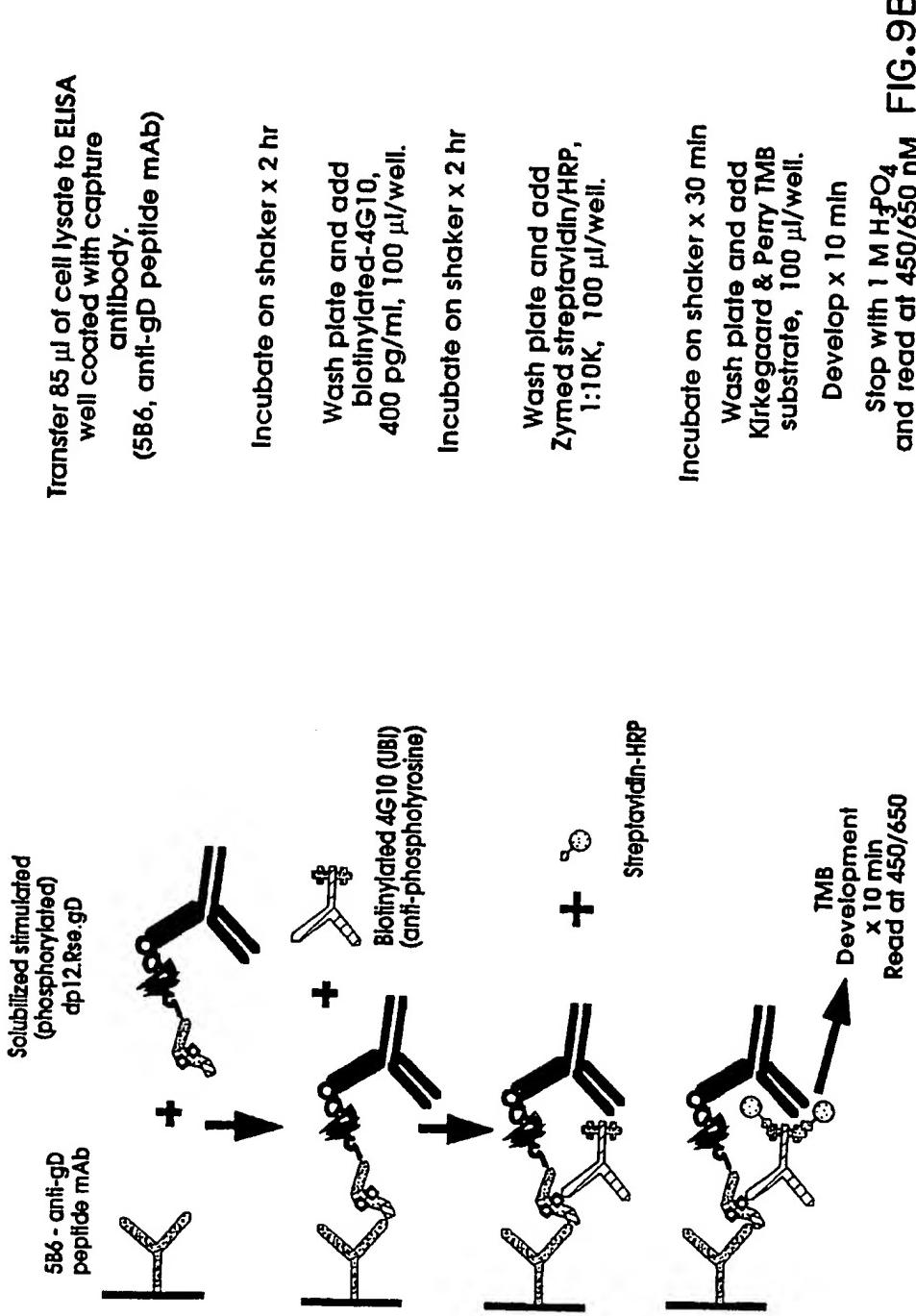
11/70



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12/70

## ELISA 96-well plate



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13/70

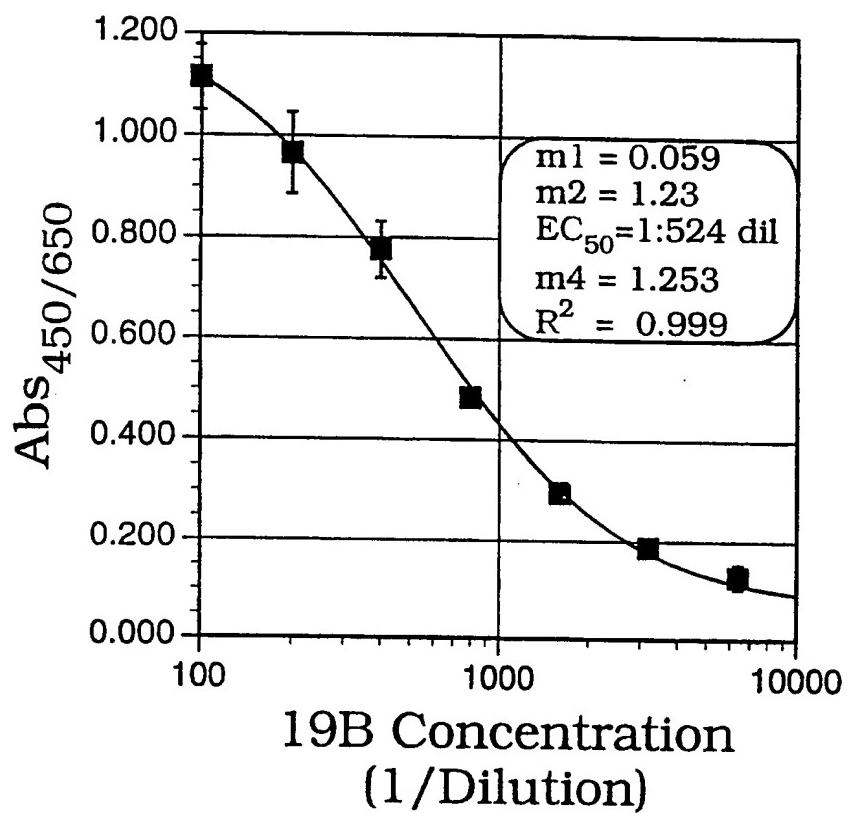
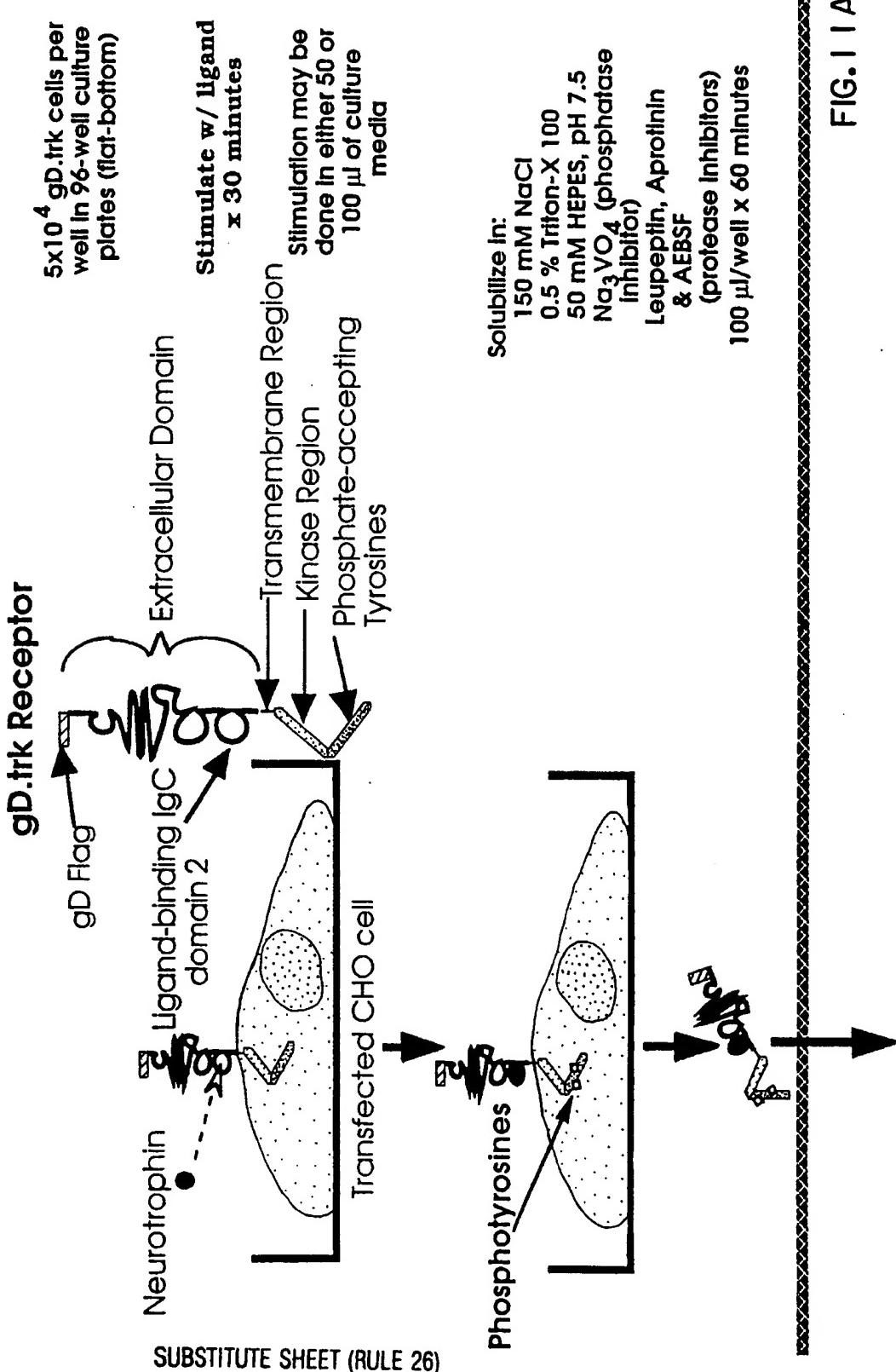


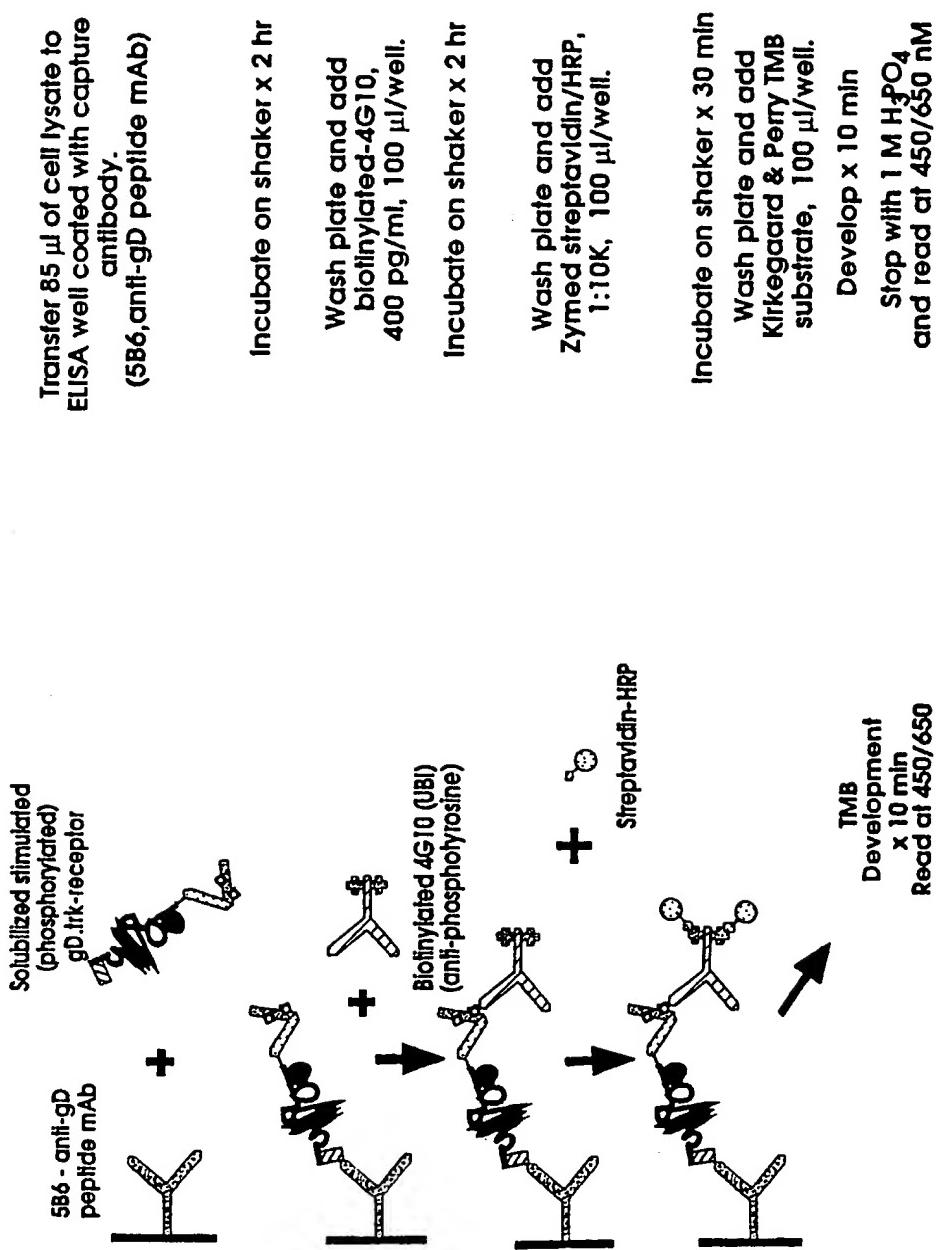
FIG. 10

14 / 70



15/70

## ELISA 96-well plate



16/70

^sp6 RNA start  
 841 TATAGAATAA CATCCACTT GCCTTTCTCT CCACAGGTGT CCACTCCAG GTCCAACCTGC  
 ATATCTTATT GTAGGTGAAA CGGAAAGAGA GGTGTCCACA GGTGAGGGTC CAGGTTGACG

^cloning linker

^R1 site mutated in

^begin gd from pchadII  
 901 ACCTGAATT CACTGCCTTC CACCAAGGCTC TGCAGGGATCC CAGAGTCAGG GGTCTGTATC  
 TGGACTTAAG GTGACGGGAAG GTGGTTCGAG ACGTCCTAGG GTCTCAGTCC CCAGACATAG

961 TTCCCTGCTGG TGGCTCCAGT TCAGGAACAG TAAACCCCTGC TCCGAATATT GCCTCTCACAC  
 AAGGACGACC ACCGAGGTCA AGTCCTTGTC ATTGGGACG AGGCTTATAA CGGAGAGTGT

1021 TCTCGTCAAT CTCCGGAGG ACTGGGGACC CTGTGACAAAG CTTCAAGGGCG AACGACCAAC  
 AGAGCACTTA GAGGCCCTCC TGACCCCTGG GACACTGTTT GAAGTGCAGG TTGCTGGTTG

1 1 ^Start gd  
 1081 TACCCCGATC ATCAGTTATC CTTAAGGTCT CTTTTGTGTG GTGCGTTCCG M\* G\* G\*  
 ATGGGGCTAG TAGTCAAATAG GAATTCCAGA GAAAACACAC CACGCAAGGC CATACCCCC  
 4 4 ^Start gd  
 1141 GACTGCCGCC AGGTTGGGG CCGTGATTTC GTTTGTCGTC ATAGTGGGCC TCCATGGGT  
 CTGACGGCGG TCCAACCCC GGCACTAAAA CAAACAGCAG TATCACCAG AGGTACCCCA

24 24 R\* G\* K\* Y\* A\* L\* A D A S L K M A D P N R F R  
 1201 CCGCGGCAA TATGCCCTGG CGGATGCCCTC TCTCAAGATG GCCGACCCCA ATCGATTTCG FIG. I 2A  
 GGGGCGCTT ATACGGAACC GCCTACGGAG AGAGTTCTAC CGGCTGGGT TAGCTAAAGC

17/70

	^Xho and GTA mutated in mature trkA																			
	G	K	D	L	P	V	L	D	O	L	L	E	V	A	A	P	C	P	D	A
44	<u>G</u>	<u>K</u>	<u>D</u>	<u>L</u>	<u>P</u>	<u>V</u>	<u>L</u>	<u>D</u>	<u>O</u>	<u>L</u>	<u>L</u>	<u>E</u>	<u>V</u>	A	A	P	C	P	D	A
1261	CGGCAAAGAC	CTTCCGGTC	TGGACCAGCT	GCTCGAGGT	GGCGCACCC	CCCCGATGC	GGCGCACCC	TGGATAGCCT	GGGGGCTACG	CGAGCTCCGA	CGAGCTGGGA	CGAGCTGGGA	CGAGCTGGGA							
64	C	C	P	H	G	S	S	G	L	R	C	T	R	D	G	A	L	D	S	L
1321	CTGCTGCC	CACGGCTCC	CGGGACTGGCG	ATGCACCCGG	GATGGGGCC	CTACCCCCGG	GTGCGAGGA	GCCCTGACGCC	TACGGGGCC	GGGACCGGGG	GGGACCGGGG	GGGACCGGGG	GGGACCGGGG							
84	H	H	L	P	G	A	E	N	L	T	E	L	Y	I	E	N	Q	Q	H	L
1381	CCACCCACCTG	CCCCGGCAG	AGAACCTGAC	TGAGCTCTAC	ATCGAGAAC	AGCAGCATCT	GGTGGTGGAC	GGGGCGCGTC	TCTTGGACTG	ACTCGAGATG	TAGCTCTTGG	TGCTCGTAGA	TGCTCGTAGA							
104	Q	H	L	E	L	R	D	L	R	G	L	G	E	L	R	N	L	T	I	V
1441	GCAGGCATCTG	GAGCTCCGTG	ATCTGAGGGG	CCTGGGGGG	CTGAGAAACC	TCACCATCGT	CGTCTAGAC	CTCGAGGCAC	TAGACTCCCC	GGACCCCTC	GACTCTTGG	AGTGGTAGCA	AGTGGTAGCA							
124	K	S	G	L	R	F	V	A	P	D	A	F	H	F	T	P	R	L	S	R
1501	GAAGAGTGGT	CTCCGTTTCG	TGGGCCAGA	TGCCTTCCAT	TTCACTCCCTC	GGCTCAGTC	CTTCTCACCA	GAGGCCAAAGC	ACCGGGTCT	ACGGAAGGTA	AAGTGGAGG	CCGAGTCAGC	CCGAGTCAGC							
144	L	N	L	S	F	N	A	L	E	S	L	S	W	K	T	V	Q	G	L	S
1561	CCTGAATCTC	TCCTTCAACG	CTCTGGAGTC	TCTCTCCTGG	AAAACTGTG	AGGGCCTCTC	GGACTTAGAG	AGGAAGTTGC	GAGACCTCAG	AGAGGGACC	TTTTGACACG	TCCC GGAGAG	TCCC GGAGAG							
164	L	Q	E	L	V	L	S	G	N	P	L	H	C	S	C	A	L	R	W	L
1621	CTTACAGGAA	CTGGTCCTGT	CGGGGAACCC	TCTGCACTGT	TCTTGACACTGT	TCTTGACACTGT	GAATGTCCCT	GACCAAGACA	GGCCCTTGGG	AGACCTGACA	AGAACACCGG	ACGGGACCCG	ACGGGACCCG							

18/70

184	Q	R	W	E	E	G	L	G	G	V	P	E	Q	K	L	Q	C	H	G	
1681	ACAGGGCTGG	GAGGAGGAGG	GA	CTGGGGCGG	A	GTGGCTGAA	C	AGAAGGCTGC	A	GTGTCATGG	T	GTCACGTACCC	T	CGACGGACTT	G	T	C	A	T	
204	Q	G	P	L	A	H	M	P	N	A	S	C	G	V	P	T	L	K	V	Q
1741	GCAAGGGCCC	CTGGCCCCACA	T	GCCCCAAT	G	CAGCTGTGGT	D	CAGGGTTACG	D	V	L	L	R	C	Q	V	E	G	TGAAGGGTCCA	
224	V	P	N	A	S	V	D	V	G	D	D	V	L	L	R	C	Q	V	E	G
1801	GGTGGCCAAT	GCCTCGGTGG	A	TGTTGGGGAA	C	GACGGTGCTG	C	GTCGACACCA	C	CACGGGTGCC	C	GTGGCTGCTG	C	TGCGGGTGGC	A	GGTGGGAGGG	T	ACTTCCAGGT		
244	R	G	L	E	Q	A	G	W	I	L	T	E	L	E	Q	S	A	T	V	M
1861	GCGGGCCTG	GAGCAGGGCCG	G	GCTGGATCCT	C	CACAGAGCTG	G	GAGCAGTCA	G	GGCAGGTGAT	G	CTGCTCGAC	G	ACGCCACGG	G	CCACGGGTGAT	T	TCCACCTCCC		
264	K	S	G	G	L	P	S	L	G	L	T	L	A	N	V	T	S	D	L	N
1921	GAAATCTGGG	GGTCTGCCAT	C	CCTGGGGCT	G	ACCCTGGGCC	A	GGCAGAGAA	C	GTGACCTCAA	G	AATGTCA	C	GTGACCTCAA	G	TGTGAGGTT	T	CACTGGAGTT		
284	R	K	N	L	T	C	W	A	E	N	D	V	G	R	A	E	V	S	V	Q
1981	CAGGAAGAAC	TTGACGTGCT	G	GGCAGAGAA	C	CGATGTGGGC	G	GGCAGAGGG	C	GGCAGAGGG	G	TCTCTGTTCA	G	TACAGTGGT	G	AGAGACAAGT	T	CACTGGAGTT		
304	V	N	V	S	F	P	A	S	V	Q	L	H	T	A	V	E	M	H	H	W
2041	GGTCAACGTC	TCCTTCCCGG	O	CAGTGTGCA	G	GTCACACAG	C	GGGTGGAGA	G	GTCACACAG	G	TGACGGTGTGC	C	GGCACCCACTG	G	FIG. 12C	T	ACGTGGTGTAC		
	CCAGTTGCAG	AGGAAGGGCC	GGTCACACGT	GGTCACACGT	GGTCACACGT	GGTCACACGT	GGTCACACGT	GGTCACACGT	GGTCACACGT	GGTCACACGT										

SUBSTITUTE SHEET (RULE 26)

19/70

324 C I P F S V D G Q P A P S L R W L F N G  
 2101 GTGCATCCC TTCTCTGTGG ATGGCAGGCC GGCAACCGTCT CTGGGCTGGC TCTTCATAATGG  
 CACGTAGGG AAGAGACACC TACCCGTGG GCGAGAAGG AGAAAGTTAAC  
  
 344 S V L N E T S F I F T E F L E P A A N E  
 2161 CTCCGTGCTC AATGAGACCA GCTTCATCTT CACTGAGTTC CTGGAGGCC CAGCCAAATGA  
 GAGGCACGAG TTACTCTGGT CGAAAGTAGAA GTGACTCAAG GACCTGGCC GTCGGTTACT  
  
 364 T V R H G C L R L N Q P T H V N N G N Y  
 2221 GACCGTGGG CACGGGTGTC TGCGCCTCAA CCAGGCCACC CACGTCAACA ACGGAAACTA  
 CTGGCACGCC GTGCCACAG ACGGCCACAG AGCGGAGTT GTTGGGTGG GTGCAGTTGT TGCCGGTGAT  
  
 384 T L A A N P F G Q A S A S I M A A F M  
 2281 CACGGTGGCTG GCTGCCAACC CCTTCGGCCA GGCCCTCGGCC TCCATCATGG CTGCCTTCAT  
 GTGGGACGAC CGACGGTTGG GGAAGCCGGT CGGGAGGGGG AGGTAGTACCGAC  
  
 404 D N P F E F N P E D P I P D T N S T S G  
 2341 GGACAACCTT TTCCGAGTTCA ACCCCGGAGGA CCCCATCCCT GACACTAAACA GCACATCTGG  
 CCTGGTGGGA AAGCTCAAGT TGGGGCTCCT GGGGTAGGGAA CTGTGATTGT CGTGTAGACCC  
  
 424 D P V E K D E T P F G V S V A V G L A  
 2401 AGACCCGGT GAGAAGAAGG ACGAAACACC TTGGGGGTC TCGGGGGCTG TGGGCTGGC  
 TCTGGCCAC CTCTTCTTC TGCTTTGTGG AAAACCCAG AGCCACCCGAC ACCCGGACCG  
  
 444 V F A C L F L S T L L L V L N K C G R R  
 2461 CGTCTTGGC TGCCCTCTTC TTTCTACGCT GCTCCTGTG GTCACAAAT GTGGACGGAG  
 GCAGAAACGG ACGGAGAAGG AAAGATGGCA CGAGGAACAC GAGTTGTTTA CACCTGGCTC

20.70

464	N	K	F	G	I	N	R	P	A	V	L	A	P	E	D	G	L	A	M	S	
2521	AAACAAAGTTT	GGGATCAACC	GCCCCGGCTGT	GCTGGCTCCA	GAGGATGGGC	TGGCCATGTC															
	TTTGTCAA	CCCTAGTTGG	GGGGCGACA	CGACCGAGGT	CTCCTACCCG	ACCGGGTACAG															
484	L	H	F	M	T	L	G	G	S	S	L	S	P	T	E	G	K	G	S	G	
2581	CCTGCATTTC	ATGACATTGG	GTGGCAGGCTC	CCITGTCCCCC	ACCGAGGGCA	AAGGCTCTGG															
	GGACGTTAAG	TACTGTAACC	CACCGTGGAG	GGACAGGGGG	TGGCTCCCGT	TTCCGAGAAC															
504	L	Q	G	H	I	I	E	N	P	Q	Y	F	S	D	A	C	V	H	H	I	
2641	GCTCCAAGGC	CACATCATCG	AGAACCCACA	ATACTTCAGT	GATGCCCTGTG	TTCACCACAT															
	CGAGGTTCCG	GTGTAGTAGC	TCTTGGGTGT	TATGAAGTCA	CTACGGACAC	AAGTGGTGT															
524	K	R	R	D	I	V	L	K	W	E	L	G	E	G	A	F	G	K	V	F	
2701	CAAGGCCGG	GACATCGTGC	TCAAGTGGGA	GCTGGGGAG	GGGGCCTTTG	GGAGGGTCTT															
	GTTCGGGGCC	CTGTAGGCACG	AGTTCACCCCT	CGACCCCTC	CGGGGAAAC	CCTTCCAGAA															
544	L	A	E	C	H	N	L	L	P	E	Q	D	K	M	L	V	A	V	K	A	
2761	CCTTGCTGAG	TGCCACAAACC	TCCGTGCCCTGA	GCAGGACAAG	ATGCTGGTGG	CTGTCAAGGC															
	GGAACGACTC	ACGGTGTGG	AGGACGGACT	CGTCCCTGTTG	TACGACCACC	GACAGTTCCG															
564	L	K	E	A	S	E	S	A	R	Q	D	F	Q	R	E	A	E	L	L	T	
2821	ACTGAAGGAG	GGGTCCGAGA	GTGCTCGGCA	GGACTTCCAA	CGTGAGGCTG	AGCTGCTCAC															
	TGACTTCCTC	CGCAGGCTCT	CACGAGCCGT	CCTGAAGGTT	GCACTCCGAC	TCGACGGAGT															
584	M	L	Q	H	Q	H	I	V	R	F	F	G	V	C	T	E	G	R	P	L	
2881	CATGCTGCAG	CACCAAGCACA	TCGTCGGCTT	CTTCGGGCTC	TGGCAGGAGG	GGGGCCCCCT															
	GTACCGACGTC	GTGGTGTGT	AGCACGGCAG	GAAGCCGCAA	ACGTGGCTCC	CGGGGGGGGA															

SUBSTITUTE SHEET (RULE 26)

**FIG. I 2E**

21/70

604	L	M	V	F	E	Y	M	R	H	G	D	L	N	R	F	L	R	S	H	G
2941	GCTCATGGTC	TTTGAGTATA	TGCGGCACGG	GGACCTAAC	CGCTTCCTCC	GATCCCATTG														
	CGAGTACAG	AAACTCATAT	ACGCCGTGCC	CCTGGAGTTG	GGAAAGGAGG	CTAGGGTACC														
624	P	D	A	K	L	L	A	G	G	E	D	V	A	P	G	P	L	G	L	G
3001	ACCTGATGCC	AAGCTGCTGG	CTGGTGGGA	GGATGTTGGCT	CAAGGCCCCC	TGGGTCTGGG														
	TGGACTACGG	TTCGACGACC	GACCACCCCT	CCTACACCGA	GGTCCGGGGG	ACCCAGACCC														
644	Q	L	L	A	V	A	S	Q	V	A	A	G	M	V	Y	L	A	G	L	H
3061	GCAGCTGCTG	GCCGTTGGCTA	GCCAGGTGCG	TGCGGGGATG	GTGTACCTGG	CGGGTCTGGCA														
	CGTCGACGAC	CGGCACCGAT	CGGTCCAGGG	ACGGCCCCAAC	ACATGGACC	GCCCAGACGT														
664	F	V	H	R	D	L	A	T	R	N	C	L	V	G	Q	G	L	V	V	K
3121	TTTTGTGCAC	CGGGACCTGG	CCACACGCAA	CTGTCTAGTG	GGCCAGGGAC	TGGTGGTCAA														
	AAAACACGTG	GCCCTGGACC	GGTGTGGTT	GACAGATCAC	CGGGTCCCTG	ACCCAGATT														
684	I	G	D	F	G	M	S	R	D	I	Y	S	T	D	Y	Y	R	V	G	G
3181	GATTGGTGTATG	TTTGGCATGA	GCAGGGATAT	CTACAGGACC	GACTATTACC	GTGTGGGAGG														
	CTAACCACTA	AAACCGTACT	CGTCCCTATA	GATGTCGTGG	CTGATAATGG	CACACCCCTCC														
704	R	T	M	L	P	I	R	W	M	P	P	E	S	I	L	Y	R	K	F	T
3241	CCGCACCATG	CTGCCCATTC	GCTGGATGCC	GCCCCGAGAGC	ATCCTGTAC	GTAAGTTCAC														
	GGCGTGGTAC	GACGGGTAAG	CGACCTACGG	CGGGCTCTCG	TAGGACATGG	CATTCAAGTG														
724	T	E	S	D	V	W	S	F	G	V	V	L	W	E	I	F	T	Y	G	K
3301	CACCGAGGCC	GACGTGTGGA	GCTTCGGCGT	GGTGTCTGG	GAGATCTCA	CCTACGGCAA														
	GTGGCTCTCG	CTGCACACCT	CGAAGCGCA	CCACGAGACC	CTCTAGAAGT	GGATGCCGTT														
744	Q	P	W	Y	Q	L	S	N	T	E	A	I	D	C	I	T	Q	G	R	E

SUBSTITUTE SHEET (RULE 26)

FIG. I 2F

22/70

3361 GCAGCCCTGG TACCAAGCTCT CCAACACCGGA GGCAATCGAC TGCATCACCGC AGGGACGTGA  
 CGTCGGGACC ATGGTCAAGA GGTGTTGCCT CCGTTAGCTG ACGTAGTGGC TCCCTGCACT

764 L E R P R A C P P E V Y A I M R G C W Q  
 3421 GTTGGAGCGG CCACGTGCCT GCCCACAGA GGTCTACGCC ATCATGCCGG GCTGCTGGCA  
 CAACCTCGCC GGTGCACCGGA CGGGTGGTCT CGAGATGCC TAGTACGCC CGACGACCGT

784 R E P Q Q R H S I K D V H A R L Q A L A  
 3481 GCGGGAGCCC CAGCAACGCC ACAGCATCAA GGATGTGCAC GCCCCGGCTGC AAGCCCTGGC  
 CGCCCTCGGG GTCCGGTGGG TGTCGTAGTT CCTACACGTG CGGGCCGACG TTGGGACCG

R1 site added with cloning primer^  
 R1 site removed with cut and fill1^

804 Q A P P V Y L D V L G O  
 3541 CCAGGCACCT CCTGTCCTACC TGGATGTCCCT GGGCTAGGAAT TAATTCAATC GATGGCCGCC  
 GGTCCGGTGGG GGACAGATGG ACCTACAGGA CCCGATCTTA ATTAAAGTTAG CTACCGGGCGG

^sv40 early poly A  
 3601 ATGGCCCAAC TTGTTTATTG CAGCTTATAA TGGTTACAAA TAAAGCAATA GCATCACAAA FIG. 12G  
 TACCGGGTTG AACAAATAAC GTCGAATATT ACCAATGTT ATTTCGTTAT CGTAGTGTGTT

23/70

^sp6 RNA start  
 841 TATAGAATAA CATCCCACTT GCCTTTCTCT CCACAGGTGT CCACTCCAG GTCCAACTRGC  
 ATATCTTATT GTAGGTGAAA CGGAAAGAGA GGTGTCCACA GGTGAGGGTC CAGGGTGACG

^cloning linker ^begin gd from pchadII  
 901 ACCTCGGTTT TATCGATTGA ATTCCACTGC CTTCCACCAA GCTCTGCAGG ATCCCAGAGT  
 TGGAGCCAAAG ATAGCTAACT TAAGGTGACG GAAGGTGGTT CGAGACGTCC TAGGGTCTCA

961 CAGGGGTCTG TATCTTCCTG CTGGTGGCTC CAGTTCAAGGA ACAGTAAACC CTGCTCCGAA  
 GTCCCCAGAC ATAGAAGGAC GACCACCGAG GTCAAGTCCCT TGTCAATTGG GACGGAGGCTT

1021 TATTGCCCTCT CACATCTCGT CAATCTCCGC GAGGACTCTGGG GACCCTGTGA CAAGCTTCAG  
 ATAACGGAGA GTGTAGAGGA GTTAGAGGGCG CTCCTGACCC CTGGGACACT GTTCGAAGTC

1081 CGCGAACGAC CAACTACCCC GATCATCAGT TATCCTTAAG GTCTCTTTG TGTGGTGGGT  
 GCGCTTGCTG GTTGATGGG CTAGTAGTCA ATAGGAATTCA CAGAGAAAC ACACCAACGCA

^Start gd  
 M\* G\* T\* A\* A\* R\* L\* G\* A\* V\* I\* L\* F\* V\* V\* I\* V\*  
 1 1141 TCCGGTATGG GGGGGACTGC CGCCAGGTG GGGGGCGTGA TTTTGTGTTGT CGTCATAGTG  
 AGGCCATACC CCCCTGACG GGGTCAAC CCCGGCACT AAACAAACA GCAGTATCAC

19 G\* L\* H\* G\* V\* R\* G\* K\* Y\* A\* L\* A D S L K M A D  
 1201 GGCTCCATG GGGTCCGGG CAAATATGCC TTGGGGATG CCTCTCTCAA GATGGCCGAC FIG. I 3A  
 CGGGAGGTAC CCCAGGGCCG GTTTATACGG AACCGCCCTAC GGAGAGTT CTACCGGGCTG

24/70

	xho and GTA mutated in^										start	mature	trkB^								
	P	N	R	F	R	G	K	D	L	P	V	L	D	O	L	L	E	V	C	P	
39																					
1261	CCC	AA	TG	GAT	TTC	GG	CA	A	AG	AC	CTT	TCG	G	C	T	GG	C	T	CC		
	GG	GT	TA	GCT	AA	GG	CG	TT	TCT	GG	AT	GG	AC	CC	CT	GG	AT	GT	CC		
59	T	S	C	K	C	S	A	S	R	I	W	C	S	D	P	S	P	G	I	V	
1321	ACG	TCC	TG	CA	AAT	GC	AG	TG	CT	CT	GG	ATC	TG	GG	AC	CC	TT	CT	CC	TG	
	GC	AA	GG	CT	GG	CT	AA	GG	CC	CT	AG	CT	AC	GT	CG	AC	GG	AT	GG	AC	
79	A	F	P	R	L	E	P	N	S	V	D	P	E	N	I	T	E	I	F	I	
1381	GC	ATT	TCC	GA	GAT	GG	AG	GC	TA	AC	AG	TG	TA	GT	GAG	GA	AC	AT	CC	TATC	
	CG	TAA	AGG	GCT	CTA	AC	TCC	G	AT	TG	TC	AC	AT	CT	AG	ACT	CT	AG	GT	G	
99	A	N	Q	K	R	L	E	I	I	N	E	D	D	V	E	A	Y	V	G	L	
1441	GCA	AA	ACC	CAG	A	AAG	GT	TAG	A	AT	CAT	AA	AC	GA	AG	AT	GAT	TG	GG	ACT	
	CG	T	TT	TT	CC	AA	TT	CT	AA	TCT	TT	AG	TT	AG	TT	GT	AG	CT	AA	AC	
119	R	N	L	T	I	V	D	S	G	L	K	F	V	A	H	K	A	F	L	K	
1501	AG	AA	AA	TCT	GA	CA	AT	TG	GG	AT	TT	GG	AT	AA	TT	TT	GG	CT	CATA	AA	
	TCT	TT	AG	ACT	G	TAA	AC	AC	CT	AAT	TT	AA	AC	AC	CC	TT	AA	AG	CT	AC	
139	N	S	N	L	Q	H	I	N	F	T	R	N	K	L	T	S	L	S	R	K	
1561	AA	CAG	CA	ACC	TG	CAG	CC	AT	TT	TAC	CA	AT	TT	AC	AA	AC	AC	TT	CT	GA	
	TG	TG	TG	GG	AC	GT	CG	TG	TT	AA	AA	AT	GG	G	CT	TT	GG	TT	AC	TG	
159	H	F	R	H	L	D	L	S	E	L	I	L	V	G	N	P	F	T	C	S	
1621	CAT	TT	CC	GT	TG	ACT	TT	GT	GA	ACT	GT	CT	GG	TTG	GCA	AT	CC	ATT	TAC	AT	GCT
	GT	AA	GG	CAG	TG	GA	AC	TG	GA	CAG	ACT	TG	AC	GT	CC	AA	CC	TT	AC	GG	

25/70

179	C	D	I	M	W	I	K	T	L	Q	E	A	K	S	S	P	D	T	Q	D
1681	TGTGACATTA	TGTGGATCAA	GACTCTCCAA	GAGGCTAAAT	CCAGTCCAGA	CACTCAGGAT														
	ACACTGTAAT	ACACCTAGTT	CTGAGGGTT	CTCCGGATTAA	GGTCAGGTCT	GTGAGTCCTA														
199	L	Y	C	L	N	E	S	S	K	N	I	P	L	A	N	L	Q	I	P	N
1741	TTGTACTGCC	TGAATGAAAG	CAGCAAGAAT	ATTCCCCCTGG	CAAACCTGCA	GATAACCCAAT														
	AACATGACGG	ACTTACTTTT	GTCGTTCTTA	TAAGGGGACC	GTTTGGACGT	CTATGGGTTA														
219	C	G	L	P	S	A	N	L	A	A	P	N	L	T	V	E	E	G	K	S
1801	TGTGGTTGCG	CATCTGCAAA	TCTGGCGCA	CCTAACCTCA	CTGTGGAGGA	AGGAAGGTCT														
	ACACCAAACG	GTAGACGTTT	AGACCGGGGT	GGATTGGAGT	GACACCTCCT	TCCTTTTCAGA														
239	I	T	L	S	C	S	V	A	G	D	P	V	P	N	M	Y	W	D	V	G
1861	ATCACATTAT	CCTGTAGTGT	GGCAGGTGAT	CCGGTTCCCTA	ATATGTATTG	GGATGTGTTGGT														
	TAGTGTAAATA	GGACATCACA	CGGTCCACTA	GGCCAAGGAT	TATACATAAC	CCTACAAACCA														
259	N	L	V	S	K	H	M	N	E	T	S	H	T	Q	G	S	L	R	I	T
1921	AACCTGGTTT	CCAAACATAT	GAATGAAACA	AGCCACACAC	AGGGCTCCTT	AAGGATAACT														
	TTGGACCAAA	GGTTTGTTATA	CTTACTTTGT	TCGGTGTGTG	TCGGGAGGAA	TCCCCTATTGA														
279	N	I	S	S	D	D	S	G	K	Q	I	S	C	V	A	E	N	L	V	G
1981	AACATTCAT	CCGATGACAG	TGGGAAGCAG	ATCTCTTGTG	TGGGGAAAAA	TCTTGTAGGA														
	TTGTAAAGTA	GGCTACTGTC	ACCCTTCGTC	TAGAGAACAC	ACCGCCTTT	AGAACATCCT														
299	E	D	Q	D	S	V	N	L	T	V	H	F	A	P	T	I	T	F	L	E
2041	GAAGATCAAG	ATTCTGTCAA	CCTCACTGTG	CATTCTGCAC	CAACTATCAC	ATTTCCTCGAA														
	CTTCTAGTT	TAAGACAGTT	GGAGTGCACAC	GTAAAACGTG	GRTGATAGTG	TAAGAGGCTT														

SUBSTITUTE SHEET (RULE 26)

FIG. I 3C

26/70

319	S	P	T	S	D	H	H	W	C	I	P	F	T	V	K	G	N	P	K	P
2101	TCTCCAACCT	CAGACCACCA	CTGGTGCATT	CCATTCACTG	TGAAAGGCAA	CCCAAAACCA														
	AGAGGTTGGA	GTCTGGGT	GACCACGTA	GTAAAGTGAC	ACTTTCGCT	GGTTTTGGT														
339	A	L	Q	W	F	Y	N	G	A	I	L	N	E	S	K	Y	I	C	T	K
2161	GCGCTTCAGT	GGTTCTATAA	CGGGGCAATA	TTGAATGAGT	CCAATACAT	CTGTACTAAA														
	CGCGAAGTCA	CCAAGATATT	GCCCCGTTAT	AACTTACTCA	GGTTATGTA	GACATGATT														
359	I	H	V	T	N	H	T	E	Y	H	G	C	L	Q	L	D	N	P	T	H
2221	ATACATGTTA	CCAATCACAC	GGAGTACAC	GGCTGCCCTCC	AGCTGGATAA	TCCCAC														
	TATGTACAAT	GGTTAGTGTG	CCTCATGGT	CCGACGGAGG	TGACACTATT	AGGGTGAGTG														
379	M	N	N	G	D	Y	T	L	I	A	K	N	E	Y	G	K	D	E	K	Q
2281	ATGAACAAATG	GGGACTACAC	TCTAAATAGCC	AAGAATGAGT	ATGGGAAGGA	TGAGAAACAG														
	TACTTGTAC	CCCTGTATGTG	AGATTATCGG	TTCTTACTCA	TACCCTTCCCT	ACTCTTTGTC														
399	I	S	A	H	F	M	G	W	P	G	I	D	D	G	A	N	P	N	Y	P
2341	ATTTCATGCTC	ACTTCATGGG	CTGGCCTGG	ATTGACCGATG	GTGCAAAACCC	AAATTATCCT														
	TAAAGACGAG	TGAAGTACCC	GACCGGACCT	TAACTGCTAC	CACGTTGGG	TTAAATAGGA														
419	D	V	I	Y	E	D	Y	G	T	A	A	N	D	I	G	D	T	T	N	R
2401	GATGTAATT	ATGAAGGATA	TGGAACGTGCA	GGAAATGACA	TGGGGGACAC	CACGAACAGA														
	CTACATTAAA	TACTTCTAAAT	ACCTTGTACGT	CGCTTACTGT	AGCCCCTGTG	GTGCTTGTCT														
439	S	N	E	I	P	S	T	D	V	T	D	K	T	G	R	E	H	L	S	V
2461	AGTAATGAAA	TCCCTTCCAC	AGACGTCACT	GATAAAACCG	GTGCGGAACA	TCTCTCGGTC														
	TCATTACTTT	AGGGAAAGGTG	TCTGGCAGTGA	CTATTTGGC	CAGCCCTGT	AGAGAGGCCAG														
459	Y	A	V	V	V	I	A	S	V	V	G	F	C	L	V	M	L	F	L	
2521	TATGCTGTGG	TGGTGATTGC	GTCTGTGGTG	GGATTGGAAAT	TTTTGGTCTCTG	FIG. I 3D														
	ATACGACACC	ACCACTAACG	CAGACACCAC	CCTAAACGG	AAAACCATA	CGACAAAGAC														

27/70

479	L	K	L	A	R	H	S	K	F	G	M	K	G	P	A	S	V	I	S	N	
2581	CTTAAGTTGG	CAAGACACTC	CAAGTTGGC	ATGAAAGGCC	CAGCCTCCGT	TATCAGGAAT															
	GAATTCAACC	GTTCGTGAG	GTTCAAACCG	TACTTTCCGG	GTCCGGAGGCA	ATAGTCGTTA															
499	D	D	S	A	S	P	L	H	H	I	S	N	G	S	N	T	P	S	S		
2641	GATGATGACT	CTGCCAGCCC	ACTCCATCAC	ATCTCCAATG	GGAGTAACAC	TCCATCTCT															
	CTACTACTGA	GACGGTCCCC	TGAGGTAGTG	TAGAGTTAC	CCTCATTTG	AGGTAGAAGA															
519	S	E	G	G	P	D	A	V	I	I	G	M	T	K	I	P	V	I	E	N	
2701	TCGGAAGGTG	GCCCAGATGC	TGTCATTATT	GGAAATGACCA	AGATCCCCGT	CATTGAAAT															
	AGCCTTCCAC	CGGGTCTACG	ACAGTAATAA	CCTTACTGGT	TCTAGGGACA	GTAACTTTA															
539	P	Q	Y	F	G	I	T	N	S	Q	L	K	P	D	T	F	V	Q	H	I	
2761	CCCCAGTACT	TTGGCATCAC	CAACAGTCAG	CTCAAGGCCAG	ACACATTGTGT	TCTAGGCACATC															
	GGGGTCACTGA	AACCGTAGTG	GTTCGTAGTC	GAGTTGGTC	TGTGTAAACA	AGTCGTGTAG															
559	K	R	H	N	I	V	L	K	R	E	L	G	E	G	A	F	G	K	V	F	
2821	AAGGGACATA	ACATTGGTCT	GAAAAGGGAG	CTAGGGGAAG	GAGCCTTTGG	AAAAGTGTTC															
	TTCGGCTGTAT	TGTAACAAGA	CTTTTCCCCTC	GATCCCCCTC	CTGGGAAACC	TTTTTCACAAAG															
579	L	A	E	C	Y	N	L	C	P	E	Q	D	K	I	L	V	A	V	K	T	
2881	CTAGCTGAAT	GCTATAACCT	CTGTCCTGAG	CAGGACAAGA	TCTTGGTGGC	AGTGAAAGACC															
	GATCGACTTA	CGATATTGGA	GACAGGACTC	GTCCCTGTTCT	AGAACCAACCG	TCACCTTCTGG															
599	L	K	D	A	S	D	N	A	R	K	D	F	H	R	E	A	E	L	L	T	
2941	CTGAAGGATG	CCAGTGACAA	TGCACGCAAG	GACTTCACC	GTGAGGCCGA	GCTCCTGACC															
	GACTTCCTAC	GGTCACTGT	CTGAAGGTGG	CACTCCGGCT	CGAGGACTG	FIG. I 3E															

28/70

619	N	L	Q	H	E	H	I	V	K	F	Y	G	V	C	V	E	G	D	P	L	
3001	AACCTCCAGC	ATGAGCACAT	CGTCAAGTTC	TATGGGTCTC	GCCTGGAGGG	CGACCCCCTC															
	TGAGGGTGTG	TACTCGTGTG	GCAGTTCAAG	ATACCGCAGA	GGCACCTCCC	GCTGGGGAG															
639	I	M	V	F	E	Y	M	K	H	G	D	L	N	K	F	L	R	A	H	G	
3061	ATCATGGTCT	TTGAGTACAT	GAAGCATGGG	GACCTCAACA	AGTTCCCTCAG	GGCACACGGC															
	TAGTACCGA	AACTCATGTG	CTTCGTACCC	CTGGAGTTGT	TCAAGGAGTC	CCGTGTGCCG															
659	P	D	A	V	L	M	A	E	G	N	P	P	T	E	L	T	Q	S	Q	M	
3121	CCTGATGCCG	TGCTGATGGC	TGAGGGCAAC	CGGCCCACGG	AACTGACCGA	GTGCGAGATG															
	GGACTAACGGC	ACGACTACCG	ACTCCCGTGTG	GGGGGGGCC	TGACTGCGT	CAGCGTCTAC															
679	L	H	I	A	Q	Q	I	A	A	G	M	V	Y	L	A	S	Q	H	F	V	
3181	CTGCATATAG	CCCAGCAGAT	CGCCGGGGC	ATGGTCTACC	TGGCGTCCCCA	GAACATTCCG															
	GACGTATATC	GGGTCGTCTA	GGGGCCCCG	TACCAAGATGG	ACCGCAGGGT	CGTGAAGGCAC															
699	H	R	D	L	A	T	R	N	C	L	V	G	E	N	L	L	V	K	I	G	
3241	CACCGGATT	TGGCCACCCAG	GAACTGCGCTG	GTCCGGGAGA	ACTTGCTGGT	GAAATCGGG															
	GTGGCGCTAA	ACCGGTGGTC	CTTGACGGAC	CAGCCCCCTCT	TGAACGACCA	CTTTTAGCCC															
719	D	F	G	M	S	R	D	V	Y	S	T	D	Y	Y	R	V	G	G	H	T	
3301	GACTTGGGA	TGTCCCCGGG	CGTGTACAGC	ACTGACTACT	ACAGGGTCTGG	TGGCCACACA															
	CTGAAACCCCT	ACAGGGCCCT	GCACATGTG	TGACTGTGTA	TGTCCCAGCC	ACCGGTGTGT															
739	M	L	P	I	R	W	M	P	P	E	S	I	M	Y	R	K	F	T	T	E	
3361	ATGCTGCCA	TTCGCTGGAT	GCCTCCAGAG	AGCATCATGT	ACAGGAAATT	CACGACGGAA															
	TACGACGGGT	AAGGCACCTA	CGGAGGCTC	TGCTAGTACA	TGTCCTTTAA	GTGCTGCCCT															
759	S	D	V	W	S	L	G	V	V	L	W	E	I	F	T	Y	G	K	Q	P	
3421	AGCGACGTCT	GGAGCCCTGGG	GGTGGTGTG	TGGGAGATT	TCACCTATGG	CAAACAGCCC															

SUBSTITUTE SHEET (RULE 26)

FIG. I 3F

29/70

TCGCTGCAGA CCTCGGACCC CCAGCACAAAC ACCCTCTAAA AGTGGATAACC GTTTGTCCCC

779 W Y Q L S N N E V I E C I T Q G R V L Q  
 3481 TGGTACCCAGC TGTCAAAACAA TGAGGGTATA GAGTGTATCA CTCAGGGCCG AGTCCTGCAG  
 ACCATGGTCG ACAGTTGTT ACTCCACTAT CTCACATAGT GAGTCCCCGC TAGGACGTC

799 R P R T C P Q E V Y E L M L G C W Q R E  
 3541 CGACCCCGCA CGTGGCCCCA GGAGGGTAT GAGCTGTATGC TGGGGTGTGC GCAGGGAGAG  
 GCTGGGGGT GCACGGGGT CCTCCACATA CTCGACTACG ACCCCACGAC CGTCGCTCTC

819 P H M R K N I K G I H T L L Q N L A K A  
 3601 CCCACATGA GGAAGAACAT CAAGGGCATC CATAACCCTCC TTCAAGAACCT GCCCAAGGCA  
 GGGGTGTACT CCTTCTGTGA GTTCCCCGTAG GTATGGAGG AAGTCCTTGAA CGGGTTCCGT

839 S P V Y L D I L G O  
 3661 TCTCCGGTCT ACCTGGACAT TCTAGGGCTAG GGCCTTTTC CCCAGACCGA TCCTTCCAA  
 AGAGGCCAGA TGGACCTGTGA AGATCCGATC CCGGGAAAG GGTCTGGCT AGGAAGGGTT

3721 CGTACTCCTC AGACGGGCTG AGAGGATGAA CATCTTTAA CTGGCGCTGG AGGCCACAA  
 GCATGAGGAG TCTGCCGAC TCTCCTACTT GTAGAAAATT GACGGCGACC TCCGGTGGTT

half Xho half Sal site from subcloning^  
 3781 GCTGCTCTCC TTCACTCTGA CAGTATTAAAC ATCAAAGACT CGGAGAAGCT CTCGACCTGC  
 CGACGAGAGG AAGTGAGACT GTCATATTG TAGTTCTGA GGCTCTCGA GAGCTGGACG

^sv40 early poly A

3841 AGAAGCTTGG CGGCCATGGC CCAACTTGGT TATTGGAGCT TATAATGGTT ACAAAATAAG FIG. I 3G  
 TCTTCTGAACC GGCGGTACCG GTTGAACAA ATAACGTCGA ATATTACAA TGTTTATTTC

30/70

^sp6 RNA start

841 TATAAGATAA CATCCACTTT GCCTTTCTCT CCACAGGTGT CCACTCCCAG GTCCAACCTGC  
 ATATCTTATT GTAGGTGAAA CGGAAGAGA GGTGTCACAA GGTGAGGGTC CAGGTTGACG

^cloning linker

^RI site mutated in

^gD from pchadII  
 901 ACCTGAATT CACTGCCCTTC CACCAAGCTC TGCAGGGATCC CAGAGTCAGG GGTCTGTATC  
 TGGACTTAAG GTGACGGAAAG GTGGTTCGAG ACGTCAGTAGG GTCTCAGTCC CCAGACATAG

961 TTCCCTGGCTGG TGCGCTCCAGT TCAGGAACAG TAAACCCCTGC TCCGAATAATT GCCTCTCACCA  
 AAGGACGACC ACCGAGGTCA AGTCCTTGTC ATTTGGGAGC AGGCTTATAA CGGAGAGTGT

1021 TCTCGTCAAT CTCCGGAGG ACTGGGGACC CTGTGACAAG CTTCAAGGGCG AACGACCAAC  
 AGAGCAGTTA GAGGGCTCC TGACCCCTGG GACACTGTTG GAAGTCCGCGC TTGCTGGTTG

^Start

gD 1 M\* G\* G\*  
 1081 TACCCCGATC ATCAGTTATC CTTAAGGTCT CTTTGTGTGT GTGGGTTCG GTATGGGGGG  
 ATGGGGCTAG TAGTCAATAG GAATTCCAGA GAAAACACAC CACGCCAGGC CATACCCCCC

4 T\* A\* A\* R\* L\* G\* A\* V\* I\* L\* F\* V\* V\* I\* V\* G\* L\* H\* G\* V\*  
 1141 GACTGCCGCC AGGTTGGGG CCGTGTATT GTTTGTCGTC ATAGTGGGCC TCCATGGGGT  
 CTGACGGCGG TCCAACCCCC GGCACTAAAA CAAACAGCG TATCACCCGG AGGTACCCCA

gD 1

24 R\* G\* K\* Y\* A\* L\* A D A S L K M A D P N R F R  
 1201 CCGGGCAA TATGCCCTGG CGGATGCCCTC TCTCAAGATG GCGGACCCCCA ATCGATTTCG  
 GGGCCCGTTT ATACGGAACC GCCTACGGAG AGAGTTCTAC CGGCTGGGGT TAGCTAAAGC

FIG. I 4A

^Xho site and GTA mutated in  
^begin mature trkC

<b>44</b> <b>1261</b>	<u>G</u> K D L P V <u>L</u> D O L L E V C P A N C V C
	CGGCAAAGAC CTTCCGGTCC TGGACCAGCT GCTCGAGGTAA TGCCCTGCAA ATTGTGTCTG
	GGCGTTCTG GAAGGCCAGG ACCTGGTCGA CGAGCTCCAT ACGGGACGTT AACACAGAC
<b>64</b> <b>1321</b>	S K T E I N C R R P D D G N L F P L L E
	CAGCAAGGACT GAGATAAATT GCCGGGGGCC GGACGATGGG AACCTCTTCC CCCCTCTGGA
	GTCGTTCTGA CTCTAGTTAA CGGGCGCGG CCTGCTACCC TTGGAGAAGG GGGGGACCT
<b>84</b> <b>1381</b>	G Q D S G N S N G N A N I N I T D I S R
	AGGGCAGGAT TCAGGGAAACA GCAATGGAA CGCCAATATC AACATCACGG ACATCTCAAG
	TCCCGTCCATA AGTCCCCTTGT CGTTACCTT GCGGTTATAG TTGTTAGTGCC TGTAGAGTTC
<b>104</b> <b>1441</b>	N I T S I H I E N W R S L H T L N A V D
	GAATATCACT TCCATACACA TAGAGAACCTG GCGCAGTCTT CACACGCTCA ACGCCGTGGA
	CTTATAGTGA AGGTATGTGT ATCTCTTGAC CGCGTCAGAA GTGTGGGAGT TGGGCACCT
<b>124</b> <b>1501</b>	M E L Y T G L Q K L T I K N S G L R S I
	CATGGAGCTC TACACGGAC TTCAAAAGCT GACCATCAAG AACTCAGGAC TTCGGAGCAT
	GTACCTCGAG ATGTGGCCTG AAGTTTCGA CTGGTAGTTTC TTGAGTCCTG AAGCCTCGTA
<b>144</b> <b>1561</b>	Q P R A F A K N P H L R Y I N L S S N R
	TCAGGCCAGA GCCTTGGCA AGAACCCCCA TTTGGGTAT ATAACCTGT CAAGTAACCG
	AGTCGGGTCT CGGAAACGGT TCTGGGGGT AACGCATA TATTTGGACA GTTCATTGGC
<b>164</b> <b>1621</b>	L T T L S W Q L F Q T L S L R E L Q L E
	GCTCACCA ACACTCGTGGC AGCTCTTCCA GACGCTGAGT CTCGGGAAT TGCAAGTTGGA

**FIG. I 4B**

32/70

	CGAGTGGTGT	GAGGCCACCG	TCCGAAAGGT	CTGCCGACTCA	GAAGCCCTTA	ACCTCAACCT	
184	Q	N	F	F	N	C	S
1681	GCAGAACTTT	TTCAACTGCA	GCTGTGACAT	CCGGCTGGATG	CAGGCTCTGGC	AGGAGGCAGGG	
	CGTCTTGAAA	AAGTTGACGT	CGACACTGTA	GGCGACCTAC	GTCCGAGACCG	TCCCTCGTC	
204	E	A	K	L	N	S	Q
1741	GGAGGCCAAG	CTCAACAGCC	AGAACCTCTA	CTGGCATCAA	GCTGTGATGGGT	CCCAGCTTCC	
	CCTCCGGTTC	GAGTTGTCGG	TCTTGGAGAT	GACGTTAGTTA	CGACTACCGA	GGGTCGAAGG	
224	L	F	R	M	N	I	S
1801	TCTCTTCCGC	ATGAAACATCA	GTCAGTGTGA	CCTTCCCTGAG	ATCAGCGTGA	GCCACCGTCAA	
	AGAGAAGGGCG	TACTTGTAGT	CAGTCACACT	GGAAAGGACTC	TAGTCGGACT	CGGTGCAGTT	
244	L	T	V	R	E	G	D
1861	CCTGACCGTA	CGAGAGGGTG	ACAATGCTGT	TATCACTTGC	AATGGCTCTG	GATCACCCCT	
	GGACTGGCAT	GCTCTCCAC	TGTTAACGACA	ATAGTGAACG	TTAACCGAGAC	CTAGTGGGGA	
264	P	D	V	D	W	I	V
1921	TCCTGATGTT	GACTGGATAG	TCACTGGGCT	GCAGTCCATC	AACACTCACC	AGACCAATCT	
	AGGACTACAC	CTGACCTATC	AGTGACCCGA	CGTCAGGTAG	TTGTGAGTGG	TCTGGTTAGA	
284	N	W	T	N	V	H	A
1981	GAACCTGGACC	AATGTTCATG	CCATCAACTT	GACGCTGGTG	AATGTGACGA	GTGAGGACAA	
	CTTGACCTGG	TTACAAGTAC	GGTAGTTGAA	CTGGCGACAC	TTACACTGCT	CACTCCCTGTT	
304	G	F	T	L	T	C	I
2041	TGGCTTCACC	CTGACCGTGC	TTGCAGAGAA	CGTGGTGGGC	ATGAGCAATG	CCAGTGTGTC	
	ACCGAAAGTGG	GACTGGACGT	AACGTCTT	GCACCCACCG	TACTCGTTAC	GGTCACAAACG	

SUBSTITUTE SHEET (RULE 26)

FIG. I 4C

33/70

324 L T V Y Y P P R V V S L E E P E L R L E  
 2101 CCTCACTGTC TACTATCCC CACGTGGT GAGCCTGGAG GAGCCTGAGC TGCGCCTGGA  
 GGAGTGACAG ATGATAGGG GTGCCACCCA CTCGGACCTC CTCGGACTCG ACGGGGACCT

344 H C I E F V V R G N P P P T L H W L H N  
 2161 GCACTGCATC GAGTTGGTGG TCCGTGCCAA CCCCCACCA ACGCTGCACT GGCTGCACAA  
 CGTGACGTAG CTCAAACACC ACGCACCGTT GGGGGTGGT TCCGACGTGA CCGACGTGTT

364 G Q P L R E S K I I H V E Y Y Q E G E I  
 2221 TGGGCAGCCT CTGCGGGAGT CCAAGATCAT CCATGTGGAA TACTACCAAG AGGGAGAGAT  
 ACCCGTGGAA GACGGCCCTCA GTTCTAGTA GGTACACCTT ATGATGGTTC TCCCTCTCTA

384 S E G C L L F N K P T H Y N N G N Y T L  
 2281 TTCCGAGGGC TGCCTGCTCT TCAACAAGCC CACCCACTAC AACAAATGGCA ACTATAACCT  
 AAGGCTCCCG ACGGACGAGA AGTTGTTGCC GTGGGTGATG TTGTTACCGT TGATATGGGA

404 I A K N P L G T A N Q T I N G H F L K E  
 2341 CATTGCCAAA AACCCACTGG GCACAGGCCAA CCAGACCATC AATGGCCACT TCCTCAAGGA  
 GTAACCGTTT TTGGGTGACC CGTGTCCGTT GGTCCTGGTAG TTACCCGGTGA AGGAGTTCCCT

424 P F P E S T D N F I L F D E V S P T P P  
 2401 GCCCTTCCA GAGAGCACGG ATAACCTTAT CTGTTTGAC GAAGTGGACT CCACACCTCC  
 CGGGAAAGGT CTCTCGTGCC TATTGAAATA GAACAAACTG CTTCACTCAG GGTGGAGGG

^begin TM ^end ecd insert ^begin TM ^end ecd insert

444 I T V T H K P E E D T F G V S I A V G L FIG.I 4D  
 2461 TATCACTGTG ACCCACAAAC CAGAAGAAGA CACTTTGGG GTATCCATAG CAGTTGGACT

34/70

ATAGTGACAC TGGGTGTTG GTCTTCTTCT GTGAAAAACCC CATAAGTATC GTCAAACCTGA

464 A A F A C V L L V V L F V M I N K Y G R  
 2521 TGCTGCTTT GCCTGTGCC TGGTGGTGGT TCTCTTCGTC ATGATCACAA AATATGGTGC  
 ACGACGAAA CGGACACAGG ACAACCAACCA AGAGAACGAG TACTAGTGTG TTATACCAGC

484 R S K F G M K G P V A V I S G E E D S A  
 2581 ACGGTCCAAA TTTGGAATGA AGGGTCCCCGT GGCTGTCTATC AGTGGTGGGG AGGACTCAAGC  
 TGCCAGGTT AACCTTACT TCCCAGGGCA CGGACAGTAG TCACCACTCC TCCTGAGTGC

504 S P L H H I N H G I T T P S S L D A G P  
 2641 CAGCCCACTG CACCACATCA ACCACGGCAT CACCACGCC TCGTCACCTGG ATGCCGGGCC  
 GTCGGGTGAC GTGGTGTAGT TGGTGGCGTA GTGGTGGGG AGCAGTGACC TACGGCCCGG

524 D T V V I G M T R I P V I E N P Q Y F R  
 2701 CGACACTGTG GTCATGGCA TGACTCGCAT CCCTGTCTATT GAGAACCCCC AGTACTTCCG  
 GCTGTGACAC CAGTAACCGT ACTGAGCGTA GGGACAGTAA CTCTTGGGG TCATGAAGGC

544 Q G H N C H K P D T Y V Q H I K R R D I  
 2761 TCAGGGACAC AACTGCCACA AGCCGGACAC GTATGTGCAG CACATTAAGA GGAGAGACAT  
 AGTCCCTGTG TTGACGGGTG CATAACGTC GTGTAATTCT CCTCTCTGTA

564 V L K R E L G E G A F G K V F L A E C Y  
 2821 CGTGCTGAAG CGAGAACTGG GTGAGGGAGC CTTTGGAAAG GTCTTCCTGG CGAGAGTGC TA FIG. I 4E  
 GCACGGACTTC GCTCTGACC CACTCCCTCG GAAACCTTC CAGAAGGACC GGCTCAGCAT

SUBSTITUTE SHEET (RULE 26)

35/70

584	N	L	S	P	T	K	D	K	M	L	V	A	V	K	A	L	K	D	P	T	
2881	CAACCTCAGC	CCGACCAAGG	ACAAGATGCT	TGTGGCTGTG	AAGGCCCTGA	AGGATCCCAC															
	GTTGGAGTCG	GGCTGGTTCC	TGTTCTACGA	ACACCGAACAC	TTCCGGGACT	TCCTAGGGTG															
604	L	A	A	R	K	D	F	Q	R	E	A	E	L	L	T	N	L	Q	H	E	
2941	CCTGGCTGCC	CGGAAGGATT	TCCAGAGGGA	GGCCGAGCTG	CTCACCAACC	TGAGGCATGA															
	GGACCCGACGG	GCCTTCCCTAA	AGGTCTCCCT	CGGCTCGAC	GAGTGGTGG	ACGTCGTACT															
624	H	I	V	K	F	Y	G	V	C	G	D	G	D	P	L	I	M	V	F	E	
3001	GCACATTGTC	AAGTTCTATG	GAGTGTGGGG	CGATGGGAC	CCCCTCATCA	TGGTCTTTGA															
	CGTGTAAACAG	TTCAAGATAAC	CTCACACGCC	GCTACCCCTG	GGGGAGTAGT	ACCGAAMCT															
644	Y	M	K	H	G	D	L	N	K	F	L	R	A	H	G	P	D	A	M	I	
3061	ATACATGAAG	CATGGAGACC	TGAATAAGTT	CCTCAGGGCC	CATGGGCCAG	ATGCAATGAT															
	TATGTACTTC	GTACCTCTGG	ACTTATCAA	GGAGTCCCGG	GTACCCGGTC	TACGTTACTA															
664	L	V	D	G	Q	P	R	Q	A	K	G	E	L	G	L	S	Q	M	L	H	
3121	CCTTGCGAT	GGACAGCCAC	GCCAGGCCAA	GGGTGAGCTG	GGGCTCTCCC	AAATGCTCCA															
	GGAACACCTA	CCTGTGGGTG	CGGTCCGGTT	CCCACTCGAC	CCCGAGAGGG	TTTACGAGGT															
684	I	A	S	Q	I	A	S	G	M	V	Y	L	A	S	Q	H	F	V	H	R	
3181	CATTGCCAGT	CAGATGCCCT	CGGGTATGGGT	GTACCTGGCC	TCCCAGGCACT	TTGTGGCACCG															
	GTAACGGTCA	GTCTAGCGGA	GCCCCATACCA	CATGGACCGG	AGGGTCGTGA	AACACGTTGCC															
704	D	L	A	T	R	N	C	L	V	G	A	N	L	L	V	K	I	G	D	F	
3241	AGACCTGGCC	ACCAGGAACT	GCCTGGTGG	AGCGAAATCTG	CTAGTGAAGA	TTGGGGACTT															
	TCTGGACCGG	TGGTCCCTGAA	CGGACCAACC	TCGCTTGTGAC	GATCACTTCT	AACCCCTGAA															
724	G	M	S	R	D	V	Y	S	T	D	Y	Y	R	V	G	G	H	T	M	L	

SUBSTITUTE SHEET (RULE 26)

^TK insert site

FIG. I 4F

36/70

3301 CGGCATGTCC AGAGATGTCT ACAGCACCGGA TTATTACAGG GTGGGAGGAC ACACCAGCT  
GCCGTACAGG TCTCTACAGA TGTGTGCCT AATAATGTC CACCCCTCTG TGTGGTACGA

744 P I R W M P P E S I M Y R K F T T E S D  
3361 CCCCATCGC TGGATGCCTC CTGAAAGCAT CATGTACCGG AAGTTCACTA CAGAGAGTGA  
GGGTAAGGG ACCTACGGAG GACTTTCGTA GTACATGGCC TTCAAGTGTAT GTCTCTCACT

764 V W S F G V I L W E I F T Y G K Q P W F  
3421 TGTATGGAGC TTGGGGTGA TCCTCTGGGA GATCTTCACC TATGGAAAGC AGCCATGGTT  
ACATACCTCG AAGCCCCACT AGGAGACCCCT CTAGAACGTTG ATACCTTTGC TCGGTACCAA

784 Q L S N T E V I E C I T Q G R V L E R P  
3481 CCAACTCTCA AACACGGAGG TCATTGAGTG CATTACCCAA GGTCGTGTT TGGAGGGCC  
GGTGTGAGAGT TTGTGCCTCC AGTAACCTCAC GTAATGGGT CCAGCACAAA ACCTCGCCGG

804 R V C P K E V Y D V M L G C W Q R E P Q  
3541 CCGAGTCTGC CCCAAAGAGG TGTACGATGT CATGCTGGGG TGCTGGCAGA GGGAAACCACA  
GGCTCAGAGC GGGTTCTCC ACATGGTACA GTACGACCCC ACGACCGTCT CCCTTGGTGT

824 Q R L N I K E I Y K I L H A L G K A T P  
3601 GCAGCGGTTG AACATCAAGG AGATCTACAA ATCCCTCCAT GCTTGGGA AGGCCACCCC  
CGTCGCCAAC TTGTAGTTCC TTGTAGTGTG TTAGGAGGTA CGAAACCCCT TCCGGTGGG

^stop  
R1 site removed with cut and fill^

844 I Y L D I L G O  
3661 AATCTACCTG GACATTCTTG GCTAGTGGTG GCTGGTGGTC ATGAAATTAAAT TCAATCGATG  
TTAGATGGAC CTGTAAGAAC CGATCACCAC CGACCAACAG TACTTAATTA AGTTAGCTAC

^sv40 early poly A  
3721 GCCGCCATGG CCCAACCTGT TTATTGCAGC TTATAATGGT TACAAATAAA GCAATAGCAT FIG. I 4G  
GGGGTACCA ATAACGTCG AAATATTACCA ATGTTTATT CGTTATCGTA

37/70

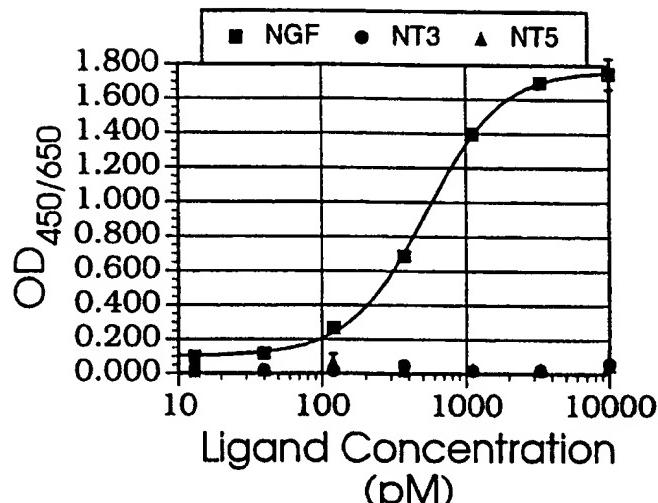
*gD.TrkA*

FIG. 15A

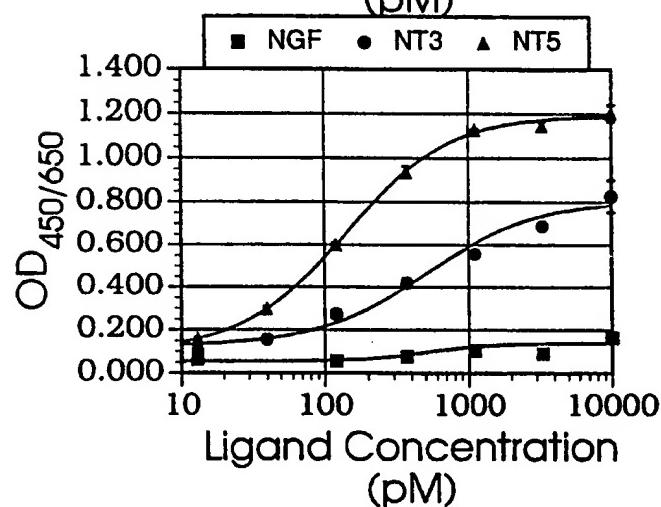
*gD.TrkB*

FIG. 15B

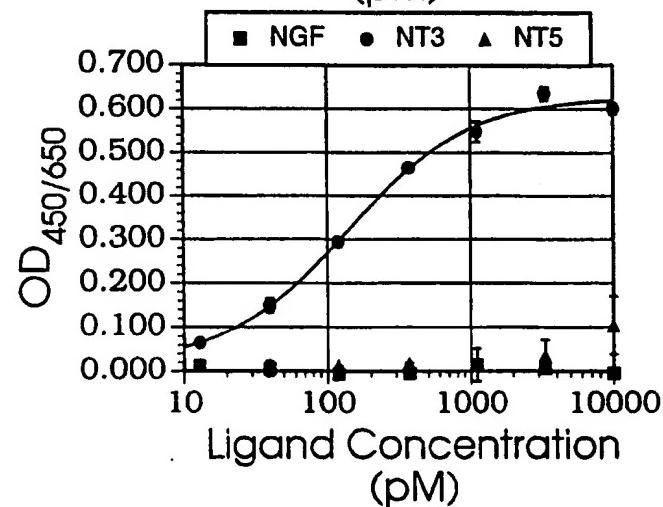
*gD.TrkC*

FIG. 15C

38/70

FIG. I 6A

39/70

scrFI	nlaIV	sfANI	ppul0I
ecoriII	mvaI	nsII/avaIII	
dsAV	bstNI	nlaIII	sphI
[dcm+]	apyI [dcm+]	nspI	nspI
141	GTTGGAAAG TCCCCAGGCT CCCCAGCAGG CAGAAGTATG CAAAGCATGC ATCTCAATT A GTCAGGAACC CACACCTTC AGGGTCCGA GGGGTCTCC GTCTCATAC GTTCGTACG TAGAGTTAAT CAGTCGTTGG	nlaIII	nlaIII
		styI	styI
		ncOI	ncOI
		bslI	bslI
		dsAI	dsAI
		aciI	aciI
		fokI	bsrI
		aciI	aciI
211	ATAGTCCGC CCCTAACTCC GCCCATCCG CCCCTAACTC CGCCCACTTC CGCCCATTC CGGCCCATG TATCAGGGCG GGGATTGAGG CGGGTAGGGC GGGGATTGAG GCGGGTCAAG GCGGGTAAGA GGCGGGGTAC	aciI	aciI
		fnu4HI	fnu4HI
		bgII	bgII
		sfiI	sfiI
		haeIII/palI	haeIII/palI
		mnlI	mnlI
		ddel	ddel
		haeIII/palI bsaJI mnlI aluI	haeIII/palI bsaJI mnlI aluI
		mnlI acII haeIII/palI	mnlI acII haeIII/palI
281	GCTGACTTAAT TTTTTTTATT TATGCAGAGG CCGAGGGCCG CTCGGCCTCT GAGCTATTCC AGAAGTAGTG CGACTGATTA AAAAAAATAA ATACGTCTCC GGCTCCGGCG GAGCCGGAGA CTCGATAAGG TCTTCATCAC	mnlI	mnlI

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**FIG. I 6B**

40/70

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41,70

541 GGGATTGGCA AGAACGGAGA CCTAACCGCTG CTTGCCTCT GGATGGACCG  
 CCCTAACCGT TCTTGCTCTT GGAGGGACCG  
 haeIII/pall  
 haeI  
 scrFI  
 mvaI bsrBI  
 ecorII  
 dsaV  
 bstNI acII  
 bsmAI apyI [dcm+] mnII ddeI asp700  
 bsaI bsauI mnII ddeI asp700  
 CCTCCGCTCA GGAACGAGTT CAAGTAATTC  
 CGAGGGAGT CCTTGCTCAA GTTCATGAAG GTTTCTTACT  
 rsaI csp6I  
 xmnI scaI  
 apyI [dcm+] mnII ddeI asp700  
 bstNI apyI [dcm+] mnII ddeI  
 scrFI  
 mvaI  
 ecorII  
 dsaV  
 bstNI apyI [dcm+] mnII ddeI  
 sexAI  
 hphI  
 alwNI  
 tflI  
 hinfl  
 eco57I  
 mboII  
 earI/ksp632I  
 mnII  
 CCACAACTC TTCACTGGAA GGTAAACAGA ATCTGGTAGT TATGGGTAGG AAAACCTGGT TCTCCATTCC  
 GGTGGAG AAGTCACCTT CCATTTGTCT TAGACCACTA ATACCCATCC TTTTGACCA AGAGGTAAGG  
 tflI  
 hinfl  
 tru9I  
 mseI  
 mboII taqI ahAI/draI aseI/asnI/vspI  
 681 TGAGAAGAAAT CGACCTTAA AGGACAGAAAT TAATATAAGT CTCAGTAGAG AACTCAAAGA ACCACCAACGA  
 ACTCTCTTA GCTGGAAATT TCCTGTCTTA ATTATCAA GAGTCATCTC TTGAGTTCTC TGGTGGTGTCT

**FIG. 16D**

42/70

**FIG. I-6E**

43/70

	nlaIII						
	sau3AI						
	mboI/ndelII [dam-]						
	dpnI [dam+]	maeII					
	dpnII [dam-]	aflIII					
	pleI	maeIII					
	hinfI	alwI [dam-]	apoI	maeIII			
891	TAGACTCTT	GTCAGCAAGGA	TCATGCCAGGA	ATTGAAAAGT	GACACGTTT	TCCCAAGAAAT	TGATTGGGG
	ATCTGAGAAA	CACTGTTCT	AGTACGTCT	TAAACTTCA	CTGTGCAGAA	AGGGTCTTTA	ACTAAACCCC
	hgAI						
	hinII/acyI						
	ahaiII/bsaHI						
	scrFI						
	mvaI	mnlI					
	ecoriI						
	dsaV						
	bstNI	econI					
	apyI [dcm+]						
	mnlI						
	bsaJI	bslI ddeI					
961	AAATATAAAC	CTCTCCCCAGA	ATACCCAGGC	GTCCTCTCTG			
	TTTATATTG	GAGGGGTCT	TATGGGTCCG	CAGGAGAGAC			

**FIG. I 6F**

44 /70

scrFI  
 mvaI  
 ecORII  
 dsav  
 bstNI  
 apyI [dcm+]  
 sau96I  
 avall  
 asuI mnII sfaNI accI mboII  
 AGGTCCAGGA GGAAAAAGGC ATCAAGTATA AGTTGAAAGT CTACGAGAAG AAAGACTAAC AGGAAGATGC  
 TCCAGGGTCC CTTTTCCG TAGTTCATAT TCAAACCTCA GATGGCTTC TTTCTGATTG TCCTCTACG  
 ^END DHFR

sfaNI  
 mboII  
 mnII  
 styI  
 ncoI  
 dsal  
 bsal  
 ppu10I  
 aluI nsII/availII  
 mnII  
 TTTCAAGTTC TCTGCTCCCC TCCTAAAGCT ATGCATTTT ATAAGACCAT GGGACTTTG  
 AAAGTTCAAG AGACGAGGG AGGATTTCGA TACGTAAAAA TATCTGGTA CCCTGAAAAC

**FIG. I 6G**

45/70

<p>styI bsaJI</p> <p>sau3AI mboI/ndeII [dam-] dpnI [dam+] dpnII [dam-] alwI [dam-] bstYI/xhoII</p> <p>1131 CTGGCTTTAG ATCCCCTTGG CTTCGTTAGA ACGGGCTAC AATTAAATACA TAACCTTATG TATCATACAC GACCGAACATC TAGGGGAACC GAAGCAATCT TGCCGCCGATG TTAATTTATGT ATTGGAAATAC ATAGTATGTG</p>	<p>fnu4HI acII thai fnDII/mvnI tru9I bstUI bsh1236I aseI/asnI/vspI</p> <p>1201 ATACGATTAA GGTGACACTA TAGATAAACAT CCACCTTGCC TTTCTCTCCA CAGGTGTCCA CTCCCCAGGT GTCACAGGT GAGGGTCCAG</p>	<p>sau96I avaII asuI scrFI mvaI ecoriI dsaV bstNI apyI [dcm+] bslI bsaJI</p> <p>maeIII hphI scfI fokI</p> <p>SUBSTITUTE SHEET (RULE 26)</p>
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**FIG. I 6H**

46/70

scrFI  
 nciI  
 mspI  
 hpaII  
 dsaV  
 xmaI/pspAI  
 smaI  
 scrFI  
 nciI  
 dsaV  
 cauII  
 bsajI  
 avaI  
 sau3AI  
 mboI/ndelI [dam-]  
 dpnI [dam+]  
 dpnII [dam-]  
 nlaIV cauII  
 bstYI/xhoII  
 bamHI bsajI  
 pleI  
 hinfi  
 taqI rmal  
 alwi [dam-]  
 scfI  
 alui pstI salI maeI alwi [dam-]  
 hindIII bspMI hincII/hindII alwi [dam-]  
 mnII  
 bsajI ddeI bsgI accI xbaI mnII bsajI  
 1271 CAACTGCACC TCGGTTCTAA GCTTCTGGAG GTCGACTCTA GAGGATCCCC  
 GTTGACGTGG AGCCAAGATT CGAAGACGTC CAGCTGAGAT CTCCCTAGGGG

**FIG. I 6!**

47/70

sau96I  
 haeIII/palI  
 acII  
 fnu4HI  
 asuI  
 bgII nlaIII  
 sfiI styI  
 eaeI ncoI  
 cfrI dsI  
 aluI  
 fnu4HI  
 bbVI  
 maeIII  
 ecORI tagI haeIII/palI  
 apoI clAI/bSP106 bsAII  
 1321 GGGGAATTCA ATCGATGCC GCCATGGCCC AACTTGTATA TTGCAGCTTA TAATGGTTAC AAATAAAGCA  
 CCCCTTAAGT TAGCTACCG CGGTACCGG TTGAAACAAAT AACGTCGAAT ATTACCAATG TTTATTTCGT  
 ^sv40

rmaI  
 bsmI maeI  
 GCATTCTAGT TGTGGTTGT CCAAACATCAT  
 TATCGTAGTG TTAAAGTGT TTATTCCGTA AAAAAGTGA CGTAAGATCA ACACCAAACA GTTGTGAGTA

**FIG. 16J**

48/70

sau3AI  
 mboI/ndelII [dam-]  
 dpnI [dam+]  
 dpnII [dam-]  
 pviI/bspCI  
 mcrI  
 taqI [dam-] tru9I  
 claiI/bsp106 [dam-]  
 sau3AI mseI  
 mboI/ndelII [dam-]  
 dpnI [dam+] xmnI  
 dpnII [dam-] aseI/asnI/vspI  
 alwI [dam-] asp700  
 nlaiI TATCATGTCT GGATCGATCG GGAATTAAATT  
 GTTACATAGA ATAGTACAGA CCTAGCTAGC CCTTAATTAA  
 sv40 origin^

1461 CAATGTATCT TATCATGTCT GGATCGATCG GGAATTAAATT  
 GTTACATAGA ATAGTACAGA CCTAGCTAGC CCTTAATTAA

haeIII/palI  
 haeI  
 styI  
 fnu4HI ncoI  
 bbVI dsalI  
 hinPI bsaJI  
 hhaI/cfoI nlaiI mnII mnII  
 CGGCGCAGCA CCATGGCCTG AAATAACCTC TGAAAGAGGA ACTTGTTAG  
 GCCGGCGTCGT GGTACCGGAC TTATTGGAG ACTTTCTCCCT TGACCCAATC CATGAAAGAC TCCGCCCTTC

1501 mnII mnII  
 acc65I ddeI acII  
 kpnI hgiCI  
 banI asp718 mnII  
 asp718 mnII  
 FIG. I 6K

49/70

**FIG. I 6L**

		nlaiV			
		scrFI			
		mvaI			
		ecoriI			
		dsav			
		bstNI			
		apyI [dcm+]			
		bsaJI			
1571	AACCAGCTGT	GGAAATGTTGTG	TCAAGTTAGGG	TGTGGAAAGT	CCCGAGGCTC
					CCCAGCAGGC
					AGAAGTATGC
	TTGGTCGACA	CCTTACACAC	AGTCAATTCC	ACACCTTCA	GGGGTCCGAG
					TCTTCATAACG
		nlaiV			
		scrFI			
		mvaI			
		ecoriI			
		dsav			
		bstNI			
		apyI [dcm+]			
		bsaJI			
1641	AAAGGCATGCCA	TCTCAATTAG	TCAGCAACCA	GGTGTGGAAA	GTCCCCAGGC
					TCCCCAGGC
					GCAGGAAGTAT
	TTTCGTAACGT	AGAGTTAAC	AGTCGTTGGT	CCACACCTT	CAGGGTCCG
					AGGGGTCTGC
					CGTCTTCATA
		nlaiV			
		scrFI			
		mvaI			
		ecoriI			
		dsav			
		bstNI			
		apyI [dcm+]			
		bsaJI			
		nlaiV			
		scrFI			
		mvaI			
		ecoriI			
		dsav			
		bstNI			
		apyI [dcm+]			
		bsaJI			
1711	GCAAAGCATG	CATCTCAATT	AGTCAGAAC	CATACTAAC	CCCTTAAC
					CGCCCATCCC
					CCCCCTAACT
	CGTTTCGTCAC	GTAGAGTTAA	TCAAGTCGTTG	GTATCAGGGC	GGGGATTGAG
					GGGGTAGGG
					CGGGGATTGAA

50/70

		nlaII		
		styI		
		ncoI		
		bsrI	bsII dsAI	mnII
		asiI	aciI bsAI	
1781	CCGCCAGT	CGCCCATT	TCCGCCCCAT	GGCTGACTAA
		GGGGGTCAA	GGCGGTAAG	AGGGGGGTA
			CCGACTGATT	AAAAAAATA AATACGTCTC
				styI
				bsaJI
				blNI
				avrII
				haeIII/palI
				stuI
				haeI
				mnII
		fnu4HI		
		bgI		
		sfiI		
		haeIII/palI		
		mnII	ddeI	
		haeIII/palI		mnII
		bsaJI	bsaJI mnII	
		aciI	haeIII/palI	mnII
1841	GCCCAGGCCG	CCTGGCCTC	TGAGCTATT	CAGAAGTAGT GAGGAGGCTT TTTGGAGGC
	CGGCTCCGGC	GGAGCCGGAG	ACTCGATAAG	GTCTTCATCA AAAACCTCCG

**FIG. I 6M**

51 / 70

acII hinPI  
 haeIII/palI hhaII/cfoI  
 mcrI thaI  
 eaGI/xmaIII/eclXI fnuDII/mvnI  
 bstUI bspMI  
 taqI eaeI hinPI scfI  
 xhoI notI hhaII/cfoI tru9I pstI  
 paeR7I CfrI tru9I ascI ahaIII/drai  
 avaI fnu4HI pacI mseI tru9I bsh1236I mseI bsgI  
 mnII acII mseI bssHII swaI sse8387I  
 rmaI maerII bsrBI fnu4HI mseI bssHII swaI sse8387I  
 maeI aluI maerII GTTAACCTCGA CGGGCCGCTT AATTAAGGCC CGCCATTAA ATCCTGCAGG  
 1901 CTAGGGCTTT GCAAAAGCT CGTTTTTCGA CAATGGAGCT CGCCGGCAA TTAAATTCCGC GCGGTAAATT TAGGACGTCC  
 GATCCGGAAA CGTTTTTCGA ^ start PUC118 ^ linearization linker inserted into HpaI site  
 scrFI  
 mvaI  
 ecORII  
 dsaV  
 bstNI  
 haeIII/palI  
 eaeI  
 cfrI maelII apyI [dcm+] tru9I  
 maeIII aluI bsRI maelII maeIII mseI  
 1971 TAACAGCTTG GCACTGGCCG TCGTTTTACA ACGTCGTGAC TGGGAAAACC CTGGCGTTAC CCAACTTAAT  
 ATTGTGAAC CGTGACCGGC AGCAAAATGT TGCGAGCACTG ACCCTTTGG GACCGCAATG GTTGAATTA

52/70

		sau3AI	
		sau96I	mboII/ndeII [dam-]
		haeIII/paII	dpnII [dam+]
		asuII	dpnII [dam-]
		aluI	mnlI
		pvuII	mboII
		nspBII	aciI
		bbvI	earI/ksp632I
		fnu4HI	mcrl
		fokI	
	2041	CACATCCCC	CTTCGCCAGC
		GCGCTTGCAG	TGGCGTAATA
		GCGAACGTC	GCGAACGGAT
		GTGTAGGGG	ACCCATTAT
		GAAGGGTGC	CGCTTCTCCG
			GGCGTGGCTA
		hinPI	
		hhAI/cfoI	
		nlaiIV	
		nari	
		kasI	
		hinII/acyI	
		hgICI	
		haeII	aciI
		banI	sfaNI
		ahaII/bsaII	sfaNI
		bgII	
	2101	CGCCCTTCCC	AACAGTTGCG
		GGGGAAAGGG	TAGCCTGAAT
		TTGTCAACGC	GGCTGATGCG
		ATCGGACTTA	GTATTCTCTC
		CCGGCTTACCG	CTTACGCATC
		CGGACTACGC	CATAAAAGAG
			GAATGGCTAG

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**FIG. I 60**

53/70

		hinPI					
		thai					
		fnuDII/mvnI					
		bstUI scfI					
		bsh1236I					
		hhAI/cfoI					
		rsaI hhAI/cfoI					
		fnu4HI					
		acII	maeII				
		fnuDII/mvnI	bstUI				
		bctUI	hhAI/cfoI				
		hinPI acII	thai				
		hhAI/cfoI	fnuDII/mvnI				
		tru9I acII	bstUI				
		mseI bsh1236I	bsh1236I				
		taATTTCGGCG CGCCCCACACC	bbvI	acII			
		2171 TTGTCGGTAT TTCACACCCG ATACGTCAA GCAACCATAAGTGCCATA	maeIII	maeIII			
		2231 ATTAAGCCGC GGGGTGTGG TGTTTACGCC CAGCGTGACC GTCTGCACTGG CGATGTGAAC	maeII	GCTACACTTG CCAGGCCCT AGCGCCCGCT GGTCGGGGAA TCGCGGGGA			
		fnu4HI	hinPI				
		thai	hhAI/cfoI				
		fnuDII/mvnI	thai				
		bctUI	hhAI/cfoI				
		hinPI acII	thai				
		hhAI/cfoI	fnuDII/mvnI				
		tru9I acII	bstUI				
		mseI bsh1236I	bsh1236I				
		taATTTCGGCG CGCCCCACACC	bbvI	acII			
		2301 CCTTTCGCTT TCTCCCTTC CTTCCTCGCC ACGTCGCC	maeII	GCTTCCCCG TCAAAGCTCTA AATCGGGGC			
		GGAAAGCGAA AGAAGGGAA GAAAGAGGAG	cfr10I	AGTTGGAGAT TTAGCCCCCG			
			nael	aluI			
			mspI				
			hpaiI				
			nael				
			mboII				
			maeII				
			aluI				
			hgiJI				
			banII				
			bmyI				
			bsp1286				
			nlaIV				

54 / 70

			mn1I				
			nlaIV				
			hg1CI				
			bani	taqI			hphI
			GGCACCTCGA	CCCCAAAAAA	CTTGATTGG		
2371	TCCCCTTAGG	GTTCCGATT	AGTGCTTAC	GGGTGGAGCT	GGGGTTTT	GAACAAACC	
	AGGAAATCC	CAAGGCTAAA	TCACGAAATG				
			maeII	haeIII/pallI			
			draiII	sau96I			
			bsaAI	asul			
2401	GTGATGGTTC	ACGTAGTGGG	CCATCGCCCT	GATAAGCAGGT	TTTCGCCCC	TTGACGTTGG	AGTCCACGTT
	CACTACAAG	TGCATCACCC	GGTAGCGGGA	CTATCTGCCA	AAAAGGGGA	AACTGCAAAC	TCAGGGCAA
			pleI				
			hinfl				
			bsrI				
2501	CTTTAATAGT	GGAECTCTGT	TCCAAACTGG	AACAAACACTC	AACCCTATCT	CGGGCTATTTC	TTTGATTAA
	GAAATTATCA	CCTGAGAACAA	AGGTGTTGACC	TTGGTGTGAG	TTGGGATAGA	GCCCCGATAAG	AAACAAATT
			bs1I				
			bs1I		avaI		
			tru9I				
			mseI				
2571	TAAGGGATT	TGCCGATTC	GGCCTATTGG	TTAAAAAAATG	AGCTGATTAA	ACAAAAAAATT	
	ATTCCCTAAA	ACGGCTAAAG	CCGGATAACC	AATTTTTAC	TCCGACTAAAT	TGTTTTAAA	
			tru9I				
			mseI				
			tru9I				
			tru9I				
			tru9I				
			apoI				
			mseI				

FIG. I 6Q

55/70

2631	AACGGCAATT	TTAACAAAT	ATTAACGTTT	ACAAATTAT	GGTGCACACT	CAGTACAATC		
	TTCGGCTTAA	AATTGTTTA	TAATTGCAA	TGTAAATA	CCACCGTGA	GTCATGTTAG		
							bsrl	hinPI
							maeIII	fnu4HI
							maeIII	nlarIII hhaI/cfoI
							bsaAI	tth111II/asPI bbVI
							ccgCTATCGCT	GTCATGGCTG CGCCCCGGACA
2691	TGCTCTGATG	CCGCATAGTT	AAGCCAACCTC	CGCTTATCGCT	ACGTGACTGG	TGCACTGACC	CAGTACCGAC	GCGGGCTGT
	ACGAGACTAC	GGCGTATCAA	TTCGGTTGAG	GGCATAGCGA	TGCACTGACC			
							sfanI	
							mspI	
							hpaII	
							scrFI	
							ncII	
							dsAV fokI	
							maeIII	
2761	acII	acII hgaI	drdI	cauII	aciI	aluI		
	CCGGCCAAACA	CCGGCTGACG	GGCCCTGACG	GGCTTGTCTG	CTCCGGCAT	CCGCTTACAG	ACAAGCTGTG	
	GGGGGGTTGT	GGGGGACTGC	GGGGGACTGC	GGGAACAGAC	GAGGGCCGTA	GGCGAATGTC	TGTTCGACAC	

**FIG. I 6R**

56 / 70

FIG. I 6S

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57/70

			bsmAI
		rcaI	
		bsrBI nlairII	
		aciI bspHI	
3001	CGGAACCCCT ATTGTTTAT TTTCCTAATT ACATTCAAAAT ATGTATCCGC TCATGAGACA ATAACCCCTGA GCCTGGGA TAAACAAATA AAAAGATTAA TGTAAGTTA TACATAGGGC AGTACTCTGT TATTGGGACT		
		mboII	
		earI/ksp632I	
3071	TAAATGCTTC AATAATATG AAAAAGGAAAG AGTATGAGTA TTCACACATT CCGTGTGCC CTTATCCCT ATTACGAAG TATTATAAC TTTTCCTTC TCATACTCAT AAGTGTAAA GCCACAGGG GAATAAGGGA		
		sspI	
		fnu4HI	
		hphI	
3141	TTTTGGGC ATTGCCCC CCTGTTTG CTCACCCAGA AACGCTGGTG AAAGTAAAAG AAAAACGGCG TAAACGGAA GGACAAAAAC GAGTGGGTCT TTGGCACAC TTTCATTTC		
		hgiAI/aspHI	
		sau3AI	
		mboI/ndelII [dam-]	
		bsp1286	
		aciI	
		bsiHKAI	
		dpnI [dam+]	
		bmyI	
		dpnII [dam-]	
		mboII [dam-]	
		apalI/snoI	
		taqI	
		alw44I/snoI	
		maeIII	
		bsrI	
		nspBII	
3201	ATGGCTGAAGA TCAGTTGGGT GCACGGAGTGG GTTACATCGA ACTGGATCTC AACAGGGTAA TAGGACTTCT AGTCAACCCA CGTGCTCACC CAATGTAGCT TGACCTAGAG TTGTGCGCCAT		

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sau3AI	mboI / ndeII [dam-]	maeII	hgIAI / aspHI
dpnI [dam+]	psp1406I	bsp1286	tru9I
dpnII [dam-]	xmnI	bsiHKAI	mseI
alwI [dam-]	asp700	bmyI	ahalII / draI
bstYI / xhoII	mboII	GATGAGCACT	TTAAAGTTC
3261 AGATCCTTGA GAGTTTTCGC CCCGAAGAAC GTTTTCCAAT CTA	TCTGTTCTTG AAAAGTTA	CTACTCGTGA AAATTCAAG	
		scrFI	
		ncII	
		mspI	
		hpall	
		dsav	
		hinII / acyI	
		hgal cauII	
		ahalI / bsaHI	
		bcgI mcrI	fnu4HI
		AGAGCAACTC	GGTCGCGCA
		TCTCGTTGAG	CCAGGGCGGT
		rsal	
		csp6I	
		bsrI	
		scal	
		hphI	maeIII
			sfanI
			fokI
ddeI	TGCTATGTGG CGCGGTATA TCCCCTGATG AGGCCGGCA	ACTCACCAGT AACCAA	CACAGAAAAG CATCTAACGG
33321	ACGATAACCC GCGCCATAAT AGGGCACTAC	TA	GTGTCTTTCA TGAGTGGTCA GTAGAATGCC
3381	TACACTATTG TCAGGAATGAC ATGTGATAAG AGTCTTACTG	AACCAA	

FIG. I 6U

59/70

		haeIII/palI		
		eaII		
		cfrI		
		fnu4HI		
3441	nlaIII ATGGCATGAC AGTAAGAGAA TTATGCAGTG TACCGTACTG	bbVI fnu4HI	nlaIII CTGCCATAAC CATGAGTGAT AACACTGCGG	acII CCAACTTACT
	TCATTCTCTT AATAACGTCA		GACGGTATTG GTACTCACTA	GGTTGAATGA
		sau96I		
		avaII		
		sau3AI mboI/ndelI	[dam-]	nlaIII sau3AI maeIII
		dpnI	[dam+]	mboI/ndelI [dam-]
		dpnII	[dam-]	dpnI
		pvuII/bspCI		[dam+]
		mcrI	aluI	dpnII [dam-]
		mnII	acII	nlaIII alwI [dam-]
		pvuII/bspCI		
		mcrI	accGCTTT	
		mnII	ttGGCACAA	
		pvuII/bspCI	ttGGGGATCA	
		mcrI	tGTAACCTGGC	
3511	TCTGACAACG ATCGGAGGAC	cgAAAGGAGCT	AACCGCTTT	
	AGACTGTTGC	GCTTCCTCCTG	ttGGCCCTGAA	
			AACGGCTGT	
			ACCCCTAGT	
			ACATTGAGCG	
		mspI		
		sau3AI mboI/ndelI	[dam-]	nlaIV fnu4HI
		dpnI	[dam+]	bsaWI bbVI
		dpnII	[dam-]	maeIII sfaNI
		ctTGATCGTT GAACTAGCAA	ggGAACCGGA CCCTTGCCCT	GGCATACCAA CGACTTACTT
				ACGACGAGCG CGGTATGGTT
				TGACACCACG ACTGTGGTC
3581				TACGGTCGTC

**FIG. I 6V**

60/70

**FIG. I 6W**

61/70

## FIG. I 6X

sau96I			pleI	
asuI			hinfI	
nlaIV				
bsrI	haeIII/palI	mnlI		
3841	ACTGGGGCCA	GATGGTAAGC	CCTCCCGTAA	CGTAGTTATC TACACGACGG GGAGTCAGGC
	TGACCCGGT	CTACCATCG	GGAGGGATA	GCATCAAATAG ATGTGCTGCC CCTCAGTCCG
ddeI				
sau3AI		nlaIV		
	mboI/ndelII [dam-]			
fokI	dpnI [dam+]	hgICl	tru9I	
3901	AACTATGGAT	GAACGAAATA	GACAGATCGC	TGAGATAGGT GCCTCACTGA TTAAGCATTG
	TTGATAACCTA	CTTGCTTAT	CTGTCTAGCG	ACTCTATCCA CGGAGTGACT ATTCTGTAAC
maeIII			tru9I	
3961	GTAACTGTCA	GACCAAGTTT	ACTCATATA	ACTTTAGATT GATTAAAAAC TTCAATTTTA
	CATTGACAGT	CTGGTCAAA	TGAGTATAA	TGAAATCTAA CTAATTTG AAGTAAAAAT
rmaI		sau3AI		
	sau3AI hphI	mboI/ndelII [dam-]		
	mboI/ndelII [dam-]			
	dpnI [dam+]	dpnI [dam+]		
	dpnII [dam-]	dpnII [dam-]		
tru9I	bstYI/xhoII	alwI [dam-]	nlaiIII	maeII
	alwI [dam-]	bstYI/xhoII		
mseI	ahaIII/draI	alwI [dam-]	rcaI	tru9I
	maeI	mboII [dam-]	bsPHI	mseI
4021	ATTTAAAAGG	ATCTAGGTGA	AGATCCTTT TGATAATCTC	ATGACCAAAA TCCCCTAACG TGAGTTCG
	TAAATTCC	TAGATCCACT	TCTAGGAAAA	ACTATTAGAG TACTGGTTT AGGGAATTGC ACTCAAAAGC

62/70

## FIG. 16Y

	sau3AI	mboI / ndeII [dam-]					
	dpnI [dam+]	sau3AI					
	dpnII [dam-]	mboI / ndeII [dam-]					
	bstyI / xholI	dpnI [dam+]					
	sau3AI	alwI [dam-]	dpnII [dam-]				
	mboI / ndeII [dam-]	alwI [dam-]	alwI [dam-]				
	dpnI [dam+]	mboII [dam-]					
	ddeI	dpnII [dam-]	bstyI / xholI				
4091	TTCCCACTGAG	CGTCAGACCC	CGTAGAAAG	ATCAAAGGAT	CTTCTTGAGA	TCCTTTTTT	
	AAGGTGACTC	GGAGTCTGGG	GCATCTTTC	TAGTTTCATA	GAAGAACTCT	AGGAAAAAA	
	thai						
	fndII/mvnI						
	bstUI						
	bsh1236I						
	hinPI	fnu4HI					
	hhAI/cf0I	bbVI					
	CTGGCGTAA	TCTGCTGCTT	GCAACACAAA	AAACCCACCGC	TACCAGCGGT	GGTTTGTGTTG	
	GACGCCATT	AGACGACGAA	CGTTTGTTT	TTGGTGGCG	ATGGTCGCCA	CCAACAAAC	
	sau3AI						
	mboI / ndeII [dam-]						
	dpnI [dam+]						
	dpnII [dam-]						
	alwI [dam-]						
	mspI	bsrI	hinPI				
	hpII	aluI	hhAI/cf0I				
4211	CCGGATCAAG	AGCTACCAAC	TCTTTTCCG	AAGGTAAC	GCTTCAGCAG	AGCGCAGATA	CCAAATACTG
	GGCCTAGTTC	TCGATGGTG	AGAAAAAGGC	TTCCATGAC	CGAAGTCGTC	TCGGTCTAT	GGTTATGAC

63/70

rmaI	bs1I	haeIII/palI						
maeI		haeI						
4281	TCCTTCTAGT	GTAGGCCGTAG	TAGGCCACC	ACTTCAAGAA	CTCTGTAGCA	CCGCCTACAT	ACCTCGCTCT	mnlI
	AGGAAGATCA	CATCGGCATC	AATCCGGTGG	TGAAGTTCTT	GAGACATCGT	GGGGATGTA	TGGAGCGAGA	
			scrFI					
			ncI					
			mspI					
			hpaII					
			dsAV		pI			
			cauII		hinFI			
			fnu4HI					
			alwNI	bbvI				
			bsRI	fnu4HI				
			maeIII	bbvI	bsRI			
			4351	GCTAATCCTG	TTACCAAGTGG	CTGCTGCCAG	TGGCGATAAAG	TCGTGTCTTA
				CGATTAGGAC	AATGGTCACC	GACGACGGTC	ACCGCTATTC	AGCACAGAAAT
			hgiAI/asPHI					
			bspBI					
			bsp1286					
			bsIKKAI					
			bmyI					
			apaLI/snoI					
			alw44I/snoI		aluI			
			maeIII	hhal/cfoI				
			4421	TAGTTACCGG	ATAAGGCCA	GCGGTGGGG	TGAACGGGG	GTTCGTGCAAC
				ATCAAATGGCC	TATTCCGGT	GGCCAGCCCC	ACTTGGCCC	ACAGCACGTG
			hinPI					
			hhaI/cfoI					
			haeII					
			ddeI	scfI				
			4491	CGACCTACAC	CGAACCTGAGA	TACCTACAGC	GTGAGGATTG	AGAAAGCGCC
				GCTGGATGTG	GCTTGACTCT	ATGGATGTG	CACTCGTAAC	ACGCTTCCCC
								AAGGGAGAAA
								TCTCCCTCTT

**FIG. I 6Z-I**

64/70

		scrFI	mvaI	
			ecoriI	mvaI
			dsav	ecoriI
			bstNI	
			bsaJI	
			hinPI mnII	
			hhAI/cfoI	
			aluI apyI [dcm+]	
4561	GCGGGACAGG TATCCGGTAA CGGCCTGTCC ATAGGCCATT CGGCCATT	acII	GCGGCAGGGT CGGAACAGGA GCCTTGTCCT	GAGGAGCAGGA GGGAGCTTCC AGGGGGAAAC CCCTCGAAGG TCCCCCTTG
		scrFI		
			dsav	
			bstNI	
			apyI [dcm+]	
4631	GCCTGGTATC TTATAGTCC TGTCGGTTT CGCACCTCT		mnII drdII hgaI	
			CGGACCATT AAATATCAGG ACAGCCCAGA CTGAACTCGC	ACTACGAGCA
			taqI	
			haeIII/palI	
			fnu4HI	
			aciI	
			thaI bsII	
			fnuDI/ mvnI	
			bstUI	
			bsh1236I	
4701	CAGGGGGCG GAGCCTATGG AAAAACGCCA GCAAACGGGC GTCCCCCGC CTCGGATAACC TTTTTGGGT CGTTGGCCG	nlaIV		
		aciI		

**FIG. 16Z-2**

65/70

	haeIII/pall			
	scrFI			
	mvaI bsII			
	ecoriI			
	dsav			
	bstNI			
	apyI [dcm+]	haeIII/pall	nspHI	
	nlalIV haeI	haeI	aflIII	
4741	CTTTTACGG	TTCTGGCCT	TTTGCTCAC	ATGTTCTTC
	CACCTATTGG	CATAATGGCG	AAACGACGG	GAAACGAGTG
	GAAAATGCC	AAGGACCGA	TACAAGAAAG	GACGGAAATAG
				GGGACTAAGA
				tfiI
				hinfi
				fnu4HI
				bbVI
				bsrBI
				aciI
				fnu4HI
				mcrl
4811	GTGGATAACC	GTATTACCGC	CTTGAGTGA	GCTGATAACCG
	CACCTATTGG	CATAATGGCG	GAAACTCACT	CTCGCCGGCAG
				CCGAACGACC
				hinPI
				haeII
				sapI hhaI/cfoI
				mbII
				earI/ksp632I
				mnII
				aciI
				acII
4871	GAGCGCAGCG	AGTCAGTGAG	CGAGGAAGCG	GAAGAGCGCC
	CTCGGTCGC	TCAGTCACTC	GCTCCTTCGC	CAATAACGCAA
				ACCGCCCTCTC
				CTTCTCGCGG
				TTTATGCCGT
				TGGCGGAGAG

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**FIG. I 6Z-3**

thaI								
fnuDII/mvnI								
bstUI								
bsh1236I								
hinPI								
hhai/cfoI								
thaI								
fnuDII/mvnI								
bstUI								
bsh1236I	haeIII/palI	tru9I	aluI					
bs1I	eaeI	t <sub>fi</sub> I	aseII/asnI/vspI	pruII				
aciI	cfrI	hinFI	mseI	nsPBI	bsrI	aciI		
4931	CCGGCCGGTT	GGCCGATTCA	TTAATCCAGC	TGGCACGACA	GTTTCCCCGA	CTGGAAAGCG		
	GGGGCGCAA	CGGGCTAAGT	ATTAGTCG	ACCGTGCTGT	CCAAAGGGCT	GACTTTCGC		
					scrFI			
					mvaI			
					ecoriI			
					dsAV			
					bsnI			
					nlaIV			
					hgICl	apyI [dcm+]		
					banI	bsaJI		
					tru9I			
					maeIII			
	hinPI	mseI						
	hhai/cfoI	aseI/asnI/vspI	mnII					
4991	GGCAGTGAGC	GCAACGCCAAT	TAATGTGAGT	TACCTCACTC	ATTAGGCACC	CCAGGCTTA	CACTTTATGC	
	CCGTCACTCG	CGTTGGTTA	ATTACACTCA	ATGGAGTGTAG	TAATCCGTGG	GGTCCGAAAT	GTGAAATAACG	

67/70

mspI		aciI		aluI	
hpAI		bsrBI		gAACAGCT	
5061 TTCCGGCTCG TATGTGTGT GGAATTGATAACA ATTTCACACA GGAAACAGCT ATGACCATGA		CCTTAACACT CGCCTATTGT TAAAGTGTG CTTTGTGCA TACTGGTACT			
tru9I					
mseI					
		aseI/asnII/vspI			
xmnI					
asp700					
5131 TTACCAATTAA A					
AATGCTTAAT T					

**FIG. I 6Z-5**

&gt;length: 5141

68/70

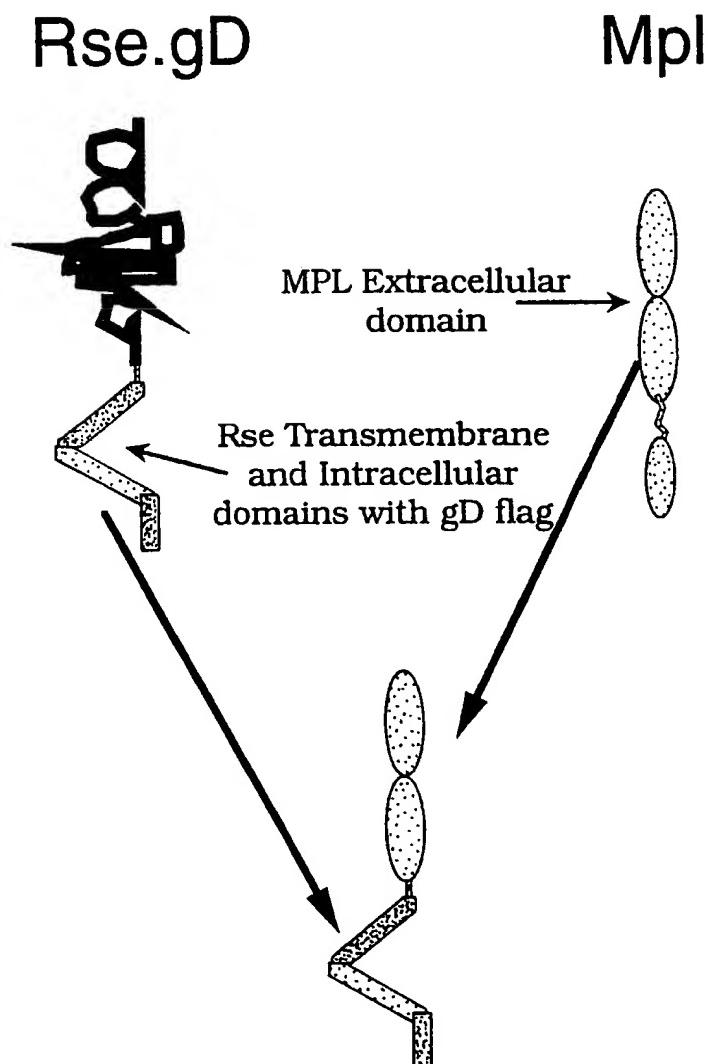
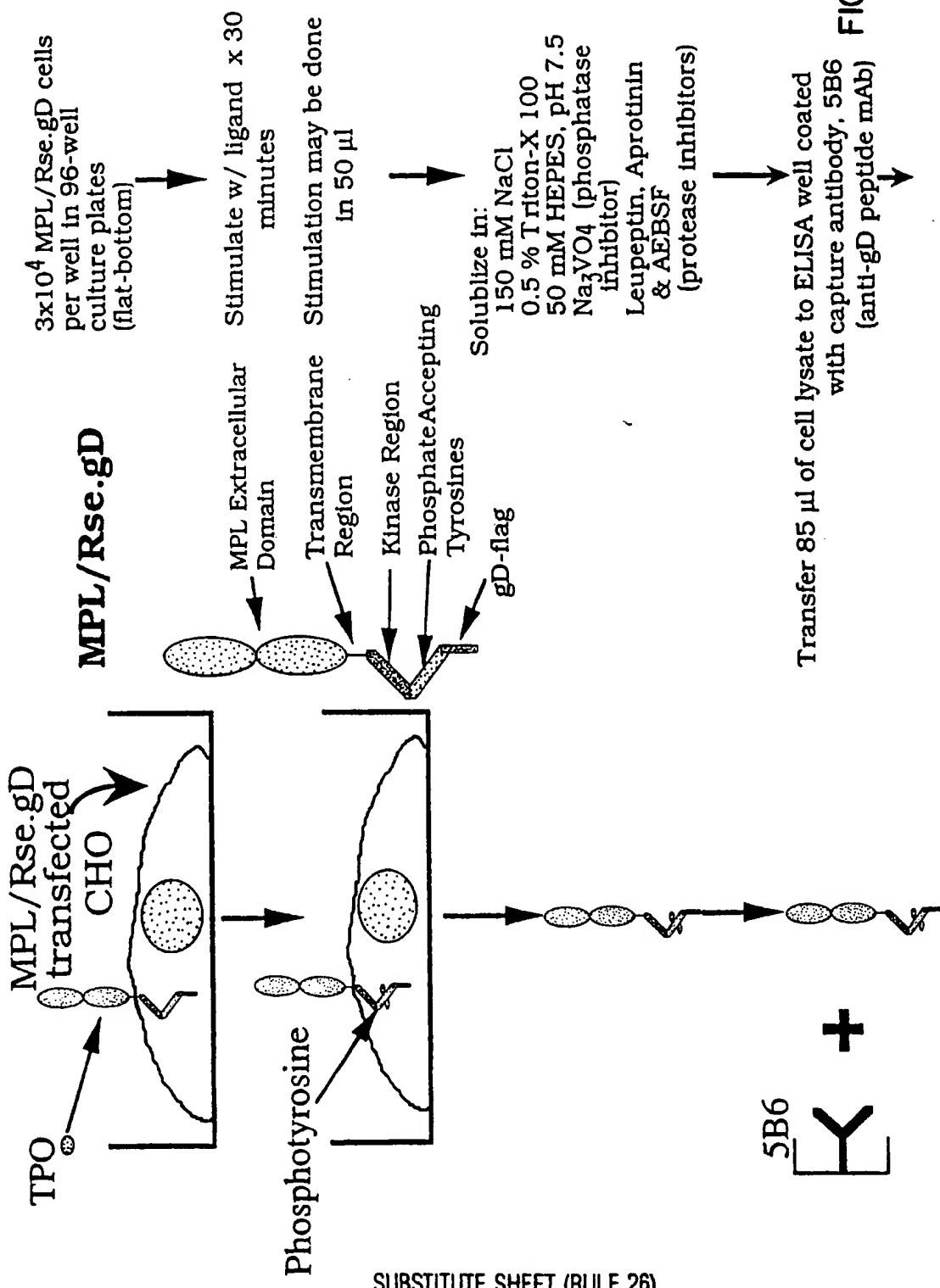


FIG. I 7

69/70



70/70

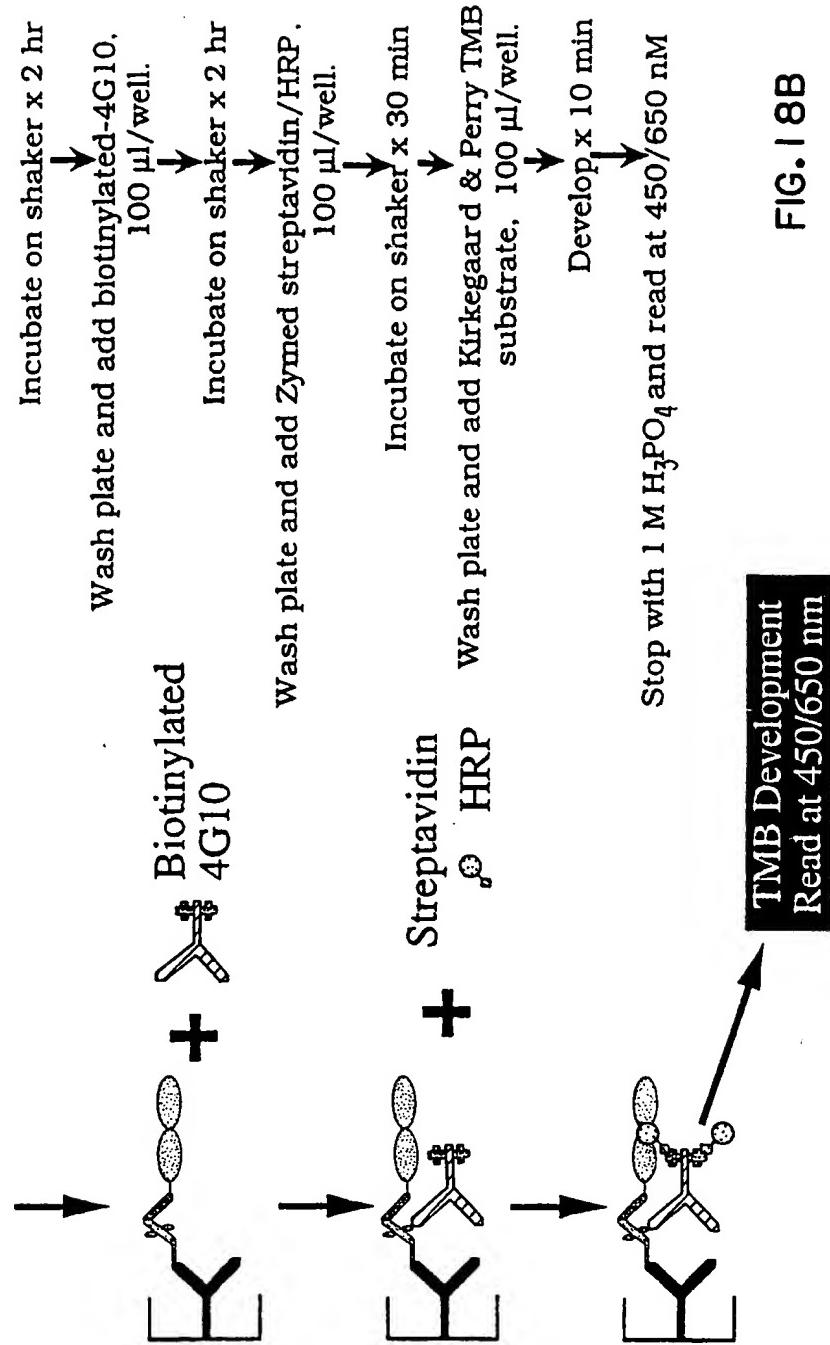


FIG. I 8B

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 94/13329

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 6 G01N33/573 G01N33/566 G01N33/543 G01N33/532 G01N33/577  
C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.269, no.14, 1 April 1994, WASHINGTON DC USA pages 10720 - 10728 M.R. MARK ET AL. 'RSE, a novel receptor-type tyrosine kinase with homology to Axl/Ufo, is expressed at high levels in the brain.' cited in the application see the whole document see the whole document ---	29-32
T	M.R. MARK ET AL. 'RSE, a novel receptor-type tyrosine kinase with homology to Axl/Ufo, is expressed at high levels in the brain.' cited in the application see the whole document see the whole document --- -/-	1-43

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Date of the actual completion of the international search

3 April 1995

Date of mailing of the international search report

27.04.95

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## INTERNATIONAL SEARCH REPORT

PCT/L 94/13329

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol.285, no.2, 1 March 1991, NEW YORK NY USA pages 197 - 204 V.P. KNUTSON ET AL. 'Comparison of insulin receptor tyrosine phosphorylation under in vitro and in situ conditions: assessment of specific protein tyrosine phosphorylation without the use of 32P-phosphate labeled substrates.' cited in the application see figure 1</p> <p>---</p>	1-28, 33-43
Y	<p>DIABETES, vol.42, 1 June 1993, WASHINGTON DC USA pages 883 - 890 H.H. KLEIN ET AL. W 'A microtiter well assay system to measure insulin activation of insulin receptor kinase in intact human mononuclear cells.' cited in the application see page 883, abstract see page 884, column 2, line 9 - line 44</p> <p>---</p>	1-28, 33-43
Y	<p>EP,A,0 244 221 (GENENTECH INCORPORATED) 4 November 1987 cited in the application see claim 1</p> <p>-----</p>	1-28, 33-43

**INTERNATIONAL SEARCH REPORT**Internatc Application No  
PCT/US 94/13329

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0244221	04-11-87	US-A-	4859609	22-08-89

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